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(54) Title: CANCER STEM CELL ANTIGEN VACCINES AND METHODS

(57) Abstract: Method of stimulating an immune response (e.g., to treat cancer) include administering to a patient a composition including dendritic cells that present cancer stem cell antigens. Compositions including cancer stem cell antigens are also provided herein.

5   ## CANCER STEM CELL ANTIGEN VACCINES AND METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS
This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/826,955, filed on September 26, 2006, the entire contents of which are incorporated herein by reference.

10   ### BACKGROUND
Over the past 30 years, a wealth of information has been generated concerning the *in vivo* and *in vitro* properties of brain tumors and rodent models of brain tumor. The 9L gliosarcoma, which was generated from inbred Fisher rats, is a widely used syngeneic rat model for brain tumors. Originally produced by N-methyl-nitrosourea mutagenesis in
15   Fisher rats, the tumor was cloned and designated 9L gliosarcoma because of its dual appearance of a glioblastoma and a sarcoma (Benda et al., J. Neurosurg., 34:310-323, 1971; Schmidek et al., J. Neurosurg., 34:335-340, 1971). The tumor could be proliferated under *in vivo* and *in vitro* conditions, making it a useful candidate as a glioma tumor model. The 9L gliosarcoma model clinically mimics rapidly growing and fatal
20   intracerebral tumors, making it the most widely used rat brain tumor model.

Stem cells have been defined as multipotent, self-renewing cells with the potential to differentiate into multiple cell types. Systems have been developed to identify the first neural stem cells in a defined media, whereby striatal embryonic progenitors could be harvested and grown in culture as undifferentiated neurospheres (clonally derived
25   aggregates of cells derived from a single stem cell) under the influence of the mitogens EGF and bFGF. Many of these cells expressed nestin (an intermediate filament found in neuroepithelial stem cells), but not markers for the more differentiated principal cell types of the CNS—neuronal and glial cells. However, when grown on coverslips treated with poly-L-ornithine, a substrate that allows cellular adhesion, many of the cells within the
30   neurospheres differentiated into neurons and astrocytes with discontinued nestin expression. The isolated striatal cells fulfilled the critical features expected from neural stem cells: an unlimited capacity for self-renewal and capacity to differentiate into the principal mature neural cells (Potten et al., Development, 110:1001-1020, 1990; Lee et al., Nat. Neurosci., 8:723-729, 2005; Maric et al., J. Neurosci., 23:240-251, 2003; Weissman
35   et al., Annu. Rev. Cell. Dev. Biol., 17:387-403, 2001; Seaberg et al., Trends Neurosci., 26:125-131, 2003; Reya et al., Nature, 414:105-111, 2001). Under similar circumstances

5   "cancer stem cells" appear to have the same characteristics of self-renewal and

multipotentcy.

Malignant brain tumors carry a poor prognosis even in the midst of surgical, radio-,

and chemotherapy. With the poor prognosis of brain tumors amidst the available

therapeutic treatments, there exists a significant need for more effective therapies to treat

10   such tumors.

SUMMARY

This invention is based, inter alia, on the discovery that vaccines based on cancer

stem cell antigens are exceptionally useful for therapy of cancer. Immunization of animals

with dendritic cells pulsed with antigens from isolated cancer stem cells provided a

15   significant survival benefit as compared to immunization with dendritic cells pulsed with

differentiated tumor cells. Cancer stem cells were found to express major

histocompatibility (MHC), indicating that they can display antigens. Further, proteins

differentially expressed in cancer stem cells as compared to differentiated tumor cells were

identified. These proteins can be useful in providing antigenic compositions for treatment

20   of cancers (e.g., neural cancers such as gliomas).

Accordingly, this application provides methods and compositions for cancer

vaccines that target cancer stem cells. Cancer stem cells are important in tumor

maintenance, proliferation, and resistance to chemotherapy and radiation therapy. Thus,

the new vaccines that target cancer stem cells provide for greater therapeutic and/or

25   prophylactic effect, particularly in cancers that are resistant to conventional treatments.

In one aspect, this application provides methods of treating cancer (e.g., neural

cancer) in a patient that include administering to the patient a composition that includes

antigen presenting cells (e.g., dendritic cells) that present cancer stem cell antigens (e.g.,

neural stem cell antigens).

30   In another aspect, this application provides methods of treating cancer (e.g., neural

cancer) in a patient that include the steps of: obtaining a population of antigen presenting

cells (e.g., dendritic cells); contacting the antigen presenting cells with a cancer stem cell

antigen composition (e.g., a neural cancer stem cell antigen composition) under conditions

such that the antigen presenting cells present cancer stem cell antigens (e.g., neural cancer

35   stem cell antigens); and administering to a patient a composition that includes the antigen

presenting cells.

5   In a further aspect, this application provides methods of inducing or stimulating an
immune response in a patient, and methods of generating antibodies specific for cancer
stem cell antigens, that include administering to the patient a composition that includes
antigen presenting cells (e.g., dendritic cells) that present cancer stem cell antigens (e.g.,
neural stem cell antigens).

10   In another aspect, this application provides methods of inducing or stimulating an
immune response in a patient, and methods of generating antibodies specific for cancer
stem cell antigens, by obtaining a population of antigen presenting cells (e.g., dendritic
cells); contacting the dendritic cells with a cancer stem cell antigen composition (e.g., a
neural cancer stem cell antigen composition) under conditions such that the antigen

15 presenting cells present cancer stem cell antigens (e.g., neural cancer stem cell antigens);
and administering to a patient a composition that includes the antigen presenting cells.

         In a further aspect, this application provides methods of preparing a cancer vaccine
(e.g., a neural cancer vaccine), that include the steps of: obtaining a population of antigen
presenting cells (e.g., dendritic cells), and contacting the antigen presenting cells with a

20 cancer stem cell antigen composition (e.g., a neural cancer stem cell antigen composition)
under conditions such that the antigen presenting cells present cancer stem cell antigens
(e.g., neural cancer stem cell antigens), thus preparing a cancer vaccine. In some
embodiments, the methods further include administering the vaccine to a patient.

         In another aspect, this application provides methods of preparing a cell vaccine for

25 treating a cancer (e.g., a neural cancer) by obtaining mononuclear cells from a subject;
culturing the mononuclear cells in vitro under conditions in which mononuclear cells
differentiate into antigen presenting cells; isolating cancer stem cells (e.g., neural cancer
stem cells) from the same or different subject; preparing a cancer stem cell antigen
composition (e.g., a neural cancer stem cell antigen composition) from the cancer stem

30 cells; and culturing the antigen presenting cells in the presence of the cancer stem cell
antigen composition, thus preparing a cell vaccine. In some embodiments, the methods
further include administering the vaccine to a patient.

         In some embodiments of any of the above aspects, the antigen presenting cells are
autologous to the subject or patient. In some embodiments, the antigen presenting cells are

35 allogeneic to the subject or patient.

         In some embodiments of any of the above aspects, the cancer stem cell antigen
composition is a lysate of cancer stem cells (e.g., neural stem cells). In other

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5   embodiments, the cancer stem cell antigen composition is an acid eluate of cancer stem
cells (e.g., neural cancer stem cells).

In some embodiments of any of the above aspects, the neural cancer stem cell
antigen composition is obtained from a brain tumor (e.g., a glioma). In some embodiments
of any of the above aspects, the cancer stem cells express CD133. In some embodiments

10   of any of the above aspects, the cancer stem cell antigen composition includes one or more
isolated peptides of CD133, CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1), maternal
embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine
phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1,
cIAP2, NAIP, or survivin. In some embodiments, the peptides are synthetic.

15   In another aspect, this application provides kits for preparing a cell vaccine for
inducing an immune response or treating a cancer (e.g., a brain cancer) that include one or
more isolated peptides of CD133, CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1),
maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine
phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1,

20   cIAP2, NAIP, or survivin.

In a further aspect, this application provides compositions (e.g.,
immunomodulatory compositions) that include antigen presenting cells (e.g., dendritic
cells) that present cancer stem cell antigens (e.g., neural cancer stem cell antigens). In
some embodiments, the cancer stem cell antigens include peptides of one or more of

25   CD133, CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1), maternal embryonic leucine
zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine phosphatase (PSP), Snail,
OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1, cIAP2, NAIP, or survivin.
In some embodiments, the compositions are produced by methods described herein.

In another aspect, this application provides for the use of compositions (e.g.,

30   immunomodulatory compositions) that include antigen presenting cells (e.g., dendritic
cells) that present cancer stem cell antigens (e.g., neural cancer stem cell antigens) in the
preparation of a medicament for modulating an immune response or treating cancer in a
subject. In some embodiments, the cancer stem cell antigens include peptides of one or
more of CD133, CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1), maternal embryonic

35   leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine phosphatase (PSP),
Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1, cIAP2, NAIP, or
survivin. In certain embodiments, the compositions are produced by methods described
herein.

5   This application also provides immunogenic compositions that include, or encode
cancer stem cell antigens, and methods of using the compositions. For example,
preparations of cancer stem cell antigens, for use as cancer vaccines (e.g., peptide
vaccines, DNA vaccines) are provided.

        "Beneficial results" may include, but are in no way limited to, lessening or
10   alleviating the severity of a disease condition, inhibiting the disease condition from
worsening, improving symptoms of a disease condition, and prolonging a patient's life or
life expectancy.

        "Cancer" and "cancerous" refer to or describe the physiological condition in
mammals that is typically characterized by unregulated cell growth. Examples of cancer
15   include, but are not limited to, breast cancer, colon cancer, lung cancer, prostate cancer,
hepatocellular cancer, gastric cancer, pancreatic cancer, cervical cancer, ovarian cancer,
liver cancer, bladder cancer, cancer of the urinary tract, thyroid cancer, renal cancer,
carcinoma, melanoma, head and neck cancer, and brain cancer; including, but not limited
to, gliomas, glioblastomas, glioblastoma multiforme (GBM), oligodendrogliomas,
20   primitive neuroectodermal tumors, low, mid and high grade astrocytomas, ependymomas
(e.g., myxopapillary ependymoma papillary ependymoma, subependymoma, anaplastic
ependymoma), oligodendrogliomas, medulloblastomas, meningiomas, pituitary adenomas,
neuroblastomas, and craniopharyngiomas.

        "Conditions" and "disease conditions," as used herein may include, but are in no
25   way limited to any form of neoplastic cell growth and proliferation, whether malignant or
benign, pre-cancerous and cancerous cells and tissues; in particular, gliomas,
glioblastomas, glioblastoma multiforme (GBM), oligodendrogliomas, primitive
neuroectodermal tumors, low, mid and high grade astrocytomas, ependymomas (e.g.,
myxopapillary ependymoma papillary ependymoma, subependymoma, anaplastic
30   ependymoma), oligodendrogliomas, medulloblastomas, meningiomas, pituitary adenomas,
neuroblastomas, and craniopharyngiomas.

        "Mammal" as used herein refers to any member of the class Mammalia, including,
without limitation, humans and nonhuman primates such as chimpanzees and other apes
and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic
35   mammals such as dogs and cats; laboratory animals including rodents such as mice, rats
and guinea pigs, and the like. The term does not denote a particular age or sex. Thus,
adult and newborn subjects, as well as fetuses, whether male or female, are intended to be

5   included within the scope of this term. The terms "patient" and "subject" are used herein interchangeably, and cover mammals, including humans.

"Pathology" of cancer includes all phenomena that compromise the well-being of the patient. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of

10   cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

"Stem-like" or "stem," as used herein refers to cells that are able to self renew from a single clone, differentiate into terminal cell types, and be serially transplantable in

15   immunodeficient animals.

"Treatment" and "treating," as used herein refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder even if the treatment is ultimately unsuccessful. Those in need of treatment include those already diagnosed as having the

20   disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. For example, in tumor (e.g., cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents or by the subject's own immune system.

"Tumor," as used herein refers to all neoplastic cell growth and proliferation,

25   whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable

30   methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference herein in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

35   Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of an exemplary process for vaccination of a patient with tumor antigen-pulsed dendritic cells. Monocytes are isolated from a patient and used to generate immature dendritic cells. After excision of a tumor or a portion of the tumor, brain tumor stem-like cells are isolated and used to prepare antigens for vaccination. The dendritic cells are pulsed with the antigens derived from the brain tumor stem-like cells. The mature antigen-presenting dendritic cells are then used to vaccinate the patient, providing an increased immune response against the tumor or residual tumor cells.

Figure 2 is a schematic diagram depicting an exemplary method of isolation and generation of tumor neurospheres.

Figures 3A-3F are a set of histograms depicting FACS analysis of neurospheres differentiated for 2 weeks. Neurospheres differentiated for 2 weeks show (3B) 96% of cells positive for the astrocyte marker GFAP; (3C) 88% of cells positive for the NSC marker nestin; (3D) 29% of cells positive for the neuronal marker MAP2; (3E) 0% of cells positive for the neuronal marker beta-tubulin III; (3F) 6% of cells positive for the oligodendrocyte marker myelin/oligodendroctye. The samples were compared to a negative control as shown (3A).

Figure 4A is a graph depicting volume of tumors induced by neurospheres (n = 4) and monolayer cells (n = 4) at day 18. Tumors induced by neurospheres were significantly larger (p < 0.02) than those from the monolayer group.

Figure 4B is a line chart depicting survival of rats with tumors induced by neurospheres (n = 9) and monolayer cells (n = 9). On average, animals in the neurosphere group died at a significantly (p < 0.02) earlier time point compared to those in the monolayer group.

Figures 5A-5E are a set of micrographs of tumors formed by implantation of neurospheres. (5A) neurospheres formed high grade gliomas with necrosis as seen on H&E; (5B) large and well circumferential tumors grew as evidenced in non-stained sections; (5C) large and well circumferential tumors grew as evidenced in sections stained for the nuclear marker DAPI; (5D) & (5E) comparison of non-tumor area (5D) and tumor area (5E) stained for reticulin reveals the high levels of reticulin formed in tumor engulfed regions, showing the histological sarcomatous component of the gliosarcoma. Scale bar = 3000 μm (5A) Scale bar = 1250 μm (5B, 5C); Scale bar = 250 μm (5D, 5E).

5　　　　　Figures 6A-6B are line graphs depicting chemoresistance of neurospheres and monolayer cells to the indicated concentrations of carboplatin (6A) and Temodar (6B). Each value represents the mean of three independent trials. Neurospheres were significantly more resistant ($p < 0.05$) to both chemotherapeutic agents as compared to monolayer cells.

10　　　　Figure 7 is a bar graph depicting proliferation rates of neurospheres and monolayer cells in serum-containing media for 2 days. The WST-1 proliferation assay was used to measure absorbance, which directly correlates to the cell number. The monolayer cells proliferated at a significantly ($p > 0.05$) greater rate than the neurosphere cells.

　　　　　Figures 8A-8C are micrographs depicting primary culture of adult human
15　glioblastoma cells. (8A) Neurosphere-like tumor cells were found in glioblastoma primary cell culture in F-12/DMEM 10% FBS medium. Scale bar = 200 µm. (8B) Neurosphere derived from a single isolated CD133$^+$ cell cultured in serum free medium with EGF/FGF. Scale bar = 200 µm. (8C) CD133 expression on a neurosphere derived from a single isolated CD133$^+$ cell in serum free medium with EGF/FGF. Staining indicates CD133
20　expression. Scale bar = 50 µm.

　　　　　Figures 9A-9C are histograms depicting CD133 protein expression on primary cultured tumor cells. Tumor cells were cultured in F-12/DMEM 10% FBS medium for 3-4 passages and stained with specific mAb to CD133 and isotype control-matched mAb. Results are given as the percentage of CD133 positive cells in the total population. In the
25　histograms, the thick lines represent staining with CD133 mAb, and the thin lines represent the isotype control-matched mAb. (9A) tumor cells from patient No. 1049; (9B) tumor cells from patient No. 377; (9C) tumor cells from patient No. 66.

　　　　　Figures 10A-10D are line graphs depicting drug sensitivity of CD133 positive cancer stem cells derived from patient No. 66. Tumor cells of No.66 were cultured in
30　FBS/F-12/DMEM medium for 4 passages. Both CD133 positive and negative tumor cells were collected by FACS sorting. 1×10^4 cells/well were plated in 96-well plate and treated with the indicated concentrations of VP-16, Taxol, temozolomide, and carboplatin for 48 hours in FBS/F-12/ DMEM medium. * indicates $p < 0.05$ compared to autologous CD133 negative cells. Data are representative of two independent experiments. (10A) VP16;
35　(10B) Taxol; (10C) temozolomide; (10D) carboplatin.

　　　　　Figures 11A-11C are a set of line graphs depicting drug sensitivity of CD133 positive cancer stem cells derived from patient Nos. 377 and 1049. Tumor cells of patient No.377 and No. 1049 were cultured in FBS/F-12/DMEM medium for 3 passages. Both

5   CD133 positive and negative tumor cells were collected by FACS sorting. 1×10^4

cells/well were plated in 96-well plate and treated with the indicated concentrations of

carboplatin (11A and 11C) or VP-16 (11B) for 48 hours in FBS/F-12/DMAEM medium. *

indicates $p < 0.05$ compared to autologous CD133 negative cells. Data are representative

of two independent experiments.

10   Figure 12 is a bar graph depicting CD133 mRNA expression in primary (P) and

recurrent (R) tumor tissue. Total RNA was extracted from both primary and recurrent

tumor tissue derived from five patients, and CD133 mRNA expression was measured by

real-time qPCR. The relative CD133 mRNA level of recurrent tumor was presented as the

fold increase compared to that of autologous primary tumor tissue. Data are representative

15   of two independent experiments.

             Figure 13 is a line graph depicting survival of 9L tumor-bearing rats vaccinated

with dendritic cells pulsed with antigens from 9L adherent cells (9L-AC), 9L daughter

cells (9L-DC), 9L neurospheres (9L-NS), and dendritic cells alone (CONTROL). Kaplan-

Meier survival curve showed that the 9L-DC group had significantly longer survival than

20   the other groups ($p = 0.0015$).

             Figure 14 is a bar graph depicting IFNγ production of splenocytes harvested from

tumor bearing rats that were re-stimulated and by either re-exposure to a 9L tumor cell

target (Target) or to no target (NO Target). The re-stimulated splenocytes from NS

vaccinated rats released high level of IFNγ when re-exposed to NS, whereas splenocytes

25   from rats vaccinated with AC or DtC had no detectable IFNγ in response to re-exposure to

AC or DC.

             Figures 15A and 15B are a pair of micrographs of tumor slices from rats vaccinated

with dendritic cells pulsed with daughter cells (17A) and neurospheres (17B) and stained

with anti-CD4 antibody. Greater infiltration of CD4^{+} cells was observed in the tumors of

30   rats vaccinated with neurosphere-pulsed dendritic cells. (Magnification ×40).

             Figure 16 is a structural diagram of CD133 depicting extracellular, intracellular,

and transmembrane regions of CD133.

             Figures 17A and 17B are cell plots and histograms depicting expression of isotype

control (17A) and MHC class I expression (17B) in human cancer stem cells.

35   Figures 18A and 18B are cell plots and histograms depicting expression of isotype

control (18A) and MHC class I expression (18B) in human neural stem cells.

DETAILED DESCRIPTION

The present application describes compositions useful as vaccines for treating cancer (e.g., neural cancers, e.g., gliomas) that include dendritic cells pulsed with antigens obtained from cancer stem cells (e.g., neurospheres); methods of producing vaccines that include dendritic cells pulsed with antigens obtained from cancer stem cells; methods of treating cancer with vaccines that include dendritic cells pulsed with antigens obtained from cancer stem cells; and kits for treating cancer that include dendritic cells pulsed with antigens obtained from cancer stem cells.

Greater tumor infiltration of cytotoxic T cells was observed in animals vaccinated against cancer stem cell antigens, and a stronger response against tumor cells was observed in T cells isolated from animals vaccinated with cancer stem cell antigens, than in responses in which antigens were not prepared from stem cell enriched cell populations. Cancer stem cells obtained from brain tumors were capable of self-renewal and proliferation, and could recapitulate the tumor when injected into rats. Isolated cancer stem cells formed more aggressive tumors as compared to differentiated tumor cells in vitro, and the cancer stem cells showed a higher resistance to chemotherapeutic agents. Similarly, CD133-positive cancer stem cells were obtained from human tumors. These cells were similarly resistant to chemotherapeutic agents and CD133-positive cells were found at a higher level in patients in whom tumors had recurred following resection.

Vaccination Methods

Described herein are methods of vaccinating a subject, e.g., to treat cancer (e.g., neural cancer, e.g., gliomas) with antigen-presenting cells ("APC"), e.g., dendritic cells ("DC"), that include antigens from cancer stem cells, e.g., presented on the surface of the antigen-presenting cells. Dendritic cells (e.g., autologous or allogeneic dendritic cells) are contacted with cancer stem cell antigens as a cell lysate, acid elution, cell extract, partially purified antigens, purified antigens, isolated antigens, partially purified peptides, purified peptides, isolated peptides, synthetic peptides, or a combination of two or more of the above. The antigen-presenting cells are then administered to a subject in need of cancer vaccination (e.g., a subject diagnosed with or at risk for cancer) to treat the cancer. Figure 1 is a schematic diagram of an exemplary process for vaccination of a patient with cancer stem cell antigen-pulsed dendritic cells.

5   <u>Cancer Stem Cells</u>

The "cancer stem cell" hypothesis proposes that only a small portion of a tumor is represented by the "cancer stem cell," which allows the tumor to proliferate and self renew, and eventually differentiate into the phenotypically diverse and heterogeneous tumor cell population (Bjerkvig et al., Nat. Rev. Cancer, 5:899-904, 2005). Cancer stem

10   cells can be isolated from any type of cancers, e.g., leukemias (Bonnet and Dick, Nat. Med., 3:730-737, 1997), breast cancers (Al-Hajj et al., Proc. Natl. Acad. Sci. USA, 100:3983-88, 2003), colon cancers (O'Brien et al., Nature, 445:106-110, 2007), and brain cancers (Singh et al., Nature, 432:396-401, 2004; Hemmati et al., Proc. Natl. Acad. Sci. USA, 100:15178-83, 2003; Singh et al., Cancer Res., 63:5821-28, 2003; Sanai et al., N.

15   Engl. J. Med., 353:811-822, 2005; Tunici et al., Mol. Cancer, 3:25, 2004). Cancer stem cells are characterized by their ability to self-renew and proliferate, and recapitulate through differentiation the tumor from which it is isolated. Additionally, neural cancer stem cells form clonally derived neurospheres in culture.

Cancer stem cells can be isolated by dissociating tumor cells and culturing them

20   under conditions that promote proliferation of stem cells (e.g., conditions that inhibit differentiation of the stem cells). Methods and conditions for isolating stem cells are known in the art. Exemplary methods and conditions can be found in U.S. Patent No. 5,589,376, U.S. Patent No. 5,643,741, U.S. Patent No. 5,650,299, U.S. Patent No. 5,824,489, U.S. Patent No. 5,849,553, U.S. Patent No. 5,928,947, U.S. Patent No.

25   5,981,708, U.S. Patent No. 6,337,184, U.S. Patent No. 6,645,763, U.S. Patent No. 6,800,790, U.S. Patent No. 6,875,607, U.S. Patent No. 6,984,522, U.S. Patent No. 7,109,03, U.S. Patent No. 7,115,267, and U.S. Patent No. 7,115,360.

Additionally, cancer stem cells can be identified or isolated (e.g., isolated from non-stem tumor cells) on the basis of expression (e.g., nucleic acid or protein expression)

30   of molecular markers, e.g., molecular markers described in the U.S. patents referred to in the above paragraph. Exemplary molecular markers include CD133, Bmi-1, Notch, Sonic hedgehog, and Wnt. Additionally, exemplary molecular markers of neural cancer stem cells include CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1), maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine phosphatase (PSP),

35   Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1, cIAP2, NAIP, and survivin.

5   Isolation or identification of cancer stem cells can be performed by standard means,
e.g., cell sorting (e.g., fluorescence activated cell sorting (FACS) or magnetic cell sorting
(MACS)).

Antigens

10   Antigenic peptides useful for loading DCs for vaccination are peptides that
stimulate a T cell mediated immune response (e.g., a cytotoxic T cell response) by
presentation to T cells on MHC molecules. Useful antigenic peptides and proteins include
those derived from cancer stem cells (e.g., neural cancer stem cells, CD133$^+$ tumor cells,
or neurospheres derived from tumors). In some embodiments, the cancer stem cell
15   antigens are presented as a lysate of the cancer stem cells. In other embodiments, the
cancer stem cell antigens are obtained by acid elution of peptides presented on MHC
molecules of the cancer stem cells. In an exemplary method, cancer stem cells are washed
with an isotonic solution (e.g., Hank's buffered saline solution) to remove media
components. The cells are then treated with acid (e.g., citrate phosphate buffer, pH 3.2) to
20   dissociate peptides from surface MHCs, and the cells removed from the solution
containing the soluble peptides. The acid-eluted cancer stem cell peptide antigens can be
further purified (e.g., on a C18 column) and frozen for storage prior to use.

Specific antigens that can be used in the methods described herein include portions
of the amino acid sequences of CD133, CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1),
25   maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine
phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1,
cIAP2, NAIP, and survivin that bind to MHC molecules and are presented to T cells.
Peptides that bind to MHC class I molecules are generally 8-10 amino acids in length.
Peptides that bind to MHC class II molecules are generally 13 amino acids or longer (e.g.,
30   13-17 amino acids long).

CD133 is a 120 kDa, five-transmembrane domain glycoprotein expressed on neural
and hematopoietic stem and progenitor cells (Yin et al., Blood, 90:5002-12, 1997). Table
1 provides an amino acid sequence of CD133 (also available in GenBank under accession
number NP_006008.1, GI:5174387). The structure of CD133 includes an extracellular N-
35   terminus, two short intracellular loops, two large extracellular loops, and an intracellular
C-terminus (FIG. 18). Exemplary CD133 T cell epitopes include 8-10 or 13-20 contiguous
amino acid residues of amino acid residues 325-350 of SEQ ID NO:53. An alternately
spliced version of CD133 is described in Yu et al., J. Biol. Chem., 277:20711-16, 2002.

5   CD90 is a cell surface glycoprotein found on T cells and neurons. Table 1 provides
an amino acid sequence of CD90 (also available in GenBank under accession number
NP_006279.2, GI:19923362).

           CD44 is a cell surface glycoprotein that may be involved in matrix adhesion. Table
1 provides an amino acid sequence of CD44 (also available in GenBank under accession
10   number NP_000601.3, GI:48255935). Several isoforms of CD44 are produced, primarily
by alternative splicing (see Marhaba et al., J. Mol. Histol., 35:211-31, 2004; Zoeller,
Cancer Immunol. Immunother., 53:567-79, 2004).

           CXCR4 is a chemokine receptor that has been found to be expressed in breast
cancers (Muller et al., Nature, 410:50-56, 2001). Table 1 provides an amino acid sequence
15   of CXCR4 (also available in GenBank under accession number NP_001008540.1,
GI:56790927). An alternative spliced variant is available in GenBank under accession
number NP_003458.1, GI:4503175.

           Nestin is an intermediate filament protein expressed in neural progenitor cells
(Dahlstrand et al., J. Cell Sci., 103:589-597, 1992). Table 1 provides an amino acid
20   sequence of Nestin (also available in GenBank under accession number NP_006608.1,
GI:38176300).

           Musashi-1 (Msi1) is an RNA-binding protein expressed in neural progenitor cells
(Good et al., Genomics, 52:382-384, 1998; Siddall et al., Proc. Nat. Acad. Sci. USA,
103:84-8407, 2006; Okano et al., Exp. Cell Res., 306:349-356, 2005). Table 1 provides an
25   amino acid sequence of Musashi-1 (also available in GenBank under accession number
NP_002433.1, GI:4505255).

           Maternal embryonic leucine zipper kinase (MELK) is a protein kinase expressed in
multiple cancers (Gray et al., Cancer Res., 65:9751-61, 2005). Table 1 provides an amino
acid sequence of MELK (also available in GenBank under accession number
30   NP_055606.1, GI:7661974).

           GLI1 is a zinc-finger transcription factor upregulated in cancers, including gliomas
(Kinzler et al., Science, 236:70-73, 1987; Kinzler et al., Nature, 332:371-374, 1988;
Kasper et al., Eur. J. Cancer, 42:437-445, 2006). Table 1 provides an amino acid sequence
of GLI1 (also available in GenBank under accession number NP_005260.1, GI:4885279).
35   PTCH1 is a transmembrane protein that is believed to function as a tumor
suppressor (Katoh et al., Cancer Biol. Ther., 4:1050-54, 2005). Table 1 provides an amino
acid sequence of PTCH1 (also available in GenBank under accession number

5 NP_000255.2, GI:134254446). Five isoforms of PTCH1 are produced by alternative
splicing (Nagao et al., Genomics, 85:462-71, 2005).

Bmi-1 is a polycomb ring finger protein involved in proliferation of progenitor
cells (Lessard et al., Nature, 423:255-260, 2003; Park et al., Nature, 423:302-305, 2003;
Molofsky et al., Nature, 425:962-967, 2003). Bmi-1 can play a role in the malignant
10 transformation of the HOX A9/MEIS-induced murine leukemia model (Lessard et al.,
Nature, 423:255-260, 2003) as well as in tumors of neural origin (van Lohuizen et al.,
Nature, 353:353-355, 1991). Table 1 provides an amino acid sequence of Bmi-1 (also
available in GenBank under accession number NP_005171.4, GI:27883842). Exemplary
Bmi-1 T cell epitopes include TLQDIVYKL (SEQ ID NO:86), CLPSPSTPV (SEQ ID
15 NO:87), VRYLETSKY (SEQ ID NO:88), KRYLRCPAA (SEQ ID NO:89),
YEEEPLKDY (SEQ ID NO:90), and KEEVNDKRY (SEQ ID NO:91) (Steele et al., Br. J.
Cancer 95:1202-11, 2006).

Phosphoserine phosphatase (PSP) is an enzyme that catalyzes the hydrolysis of O-
phosphoserine. Table 1 provides an amino acid sequence of PSP (also available in
20 GenBank under accession number NP_004568.2, GI:46249388).

Snail is a zinc-finger transcription factor and anti-apoptotic protein (Vega et al.,
Genes Dev., 18:1131-1143, 2004). Table 1 provides an amino acid sequence of Snail (also
available in GenBank under accession number NP_005976.2, GI:18765741).

OCT4 is a POU homeodomain-containing transcription factor expressed in
25 pluripotent cells (Nichols et al., Cell, 95:379-391, 1998). Table 1 provides an amino acid
sequence of OCT4 (also available in GenBank under accession number NP_002692.2,
GI:42560248). An alternate isoform of OCT4 is available in GenBank under accession
number NP_976034.3, GI:116235491.

BCRP1 is an ATP-binding cassette (ABC) transporter protein involved in
30 multidrug resistance of tumors (Doyle et al., Proc. Nat. Acad. Sci. USA, 95:1566570,
1998). Table 1 provides an amino acid sequence of BCRP1 (also available in GenBank
under accession number NP_004818.2, GI:62526033).

MGMT is an O-6-methylguanine-DNA methyltransferase DNA -mismatch repair
protein that can provide resistance to some methylating and chloroethylating agents, such
35 as temozolomide (Rabik et al., Cancer Treat. Rev., 32:261-276, 2006; Cai et al., Cancer
Res., 65:3319-27, 2005). Table 1 provides an amino acid sequence of MGMT (also
available in GenBank under accession number NP_002403.1, GI:4505177).

5   BCL-2 is a mitochondrial anti-apoptotic protein correlated with chemotherapy

resistant cancers and decreased overall survival (Campos et al., Blood, 81:3091-3096,

1993). Table 1 provides an amino acid sequence of BCL-2 (also available in GenBank

under accession number NP_000624.2, GI:72198189). An alternatively spliced isoform of

BCL-2 is available in GenBank under accession number NP_000648.2, GI:72198346.

10   FLIP is an anti-apoptotic protein (Irmler et al., Nature, 388:190-195, 1997). Table

1 provides an amino acid sequence of FLIP (also available in GenBank under accession

number NP_003870.3, GI:21361769).

BCL-XL is an anti-apoptotic protein related to BCL-2, which may be involved in

chemoresistance (Boise et al., Cell, 74:597-608, 1993; Andreeff et al., Leukemia, 13:1881-

15   92, 1999). Table 1 provides an amino acid sequence of BCL-XL (also available in

GenBank under accession number NP_612815.1, GI:20336335). Exemplary T cell

epitopes of BCL-XL include Bcl-xL118–126 (TAYQSFEQV; SEQ ID NO:92), Bcl-

xL173–182 (YLNDHLEPWI; SEQ ID NO:93), and Bcl-xL169–178 (WMATYLNDHL;

SEQ ID NO:94) (Andersen et al., J. Immunol., 175:2709-14, 2005).

20   XIAP is a member of the inhibitor of apoptosis protein (IAP) family (Deveraux et

al., Nature, 388:300-304, 1997). Table 1 provides an amino acid sequence of XIAP (also

available in GenBank under accession number NP_001158.2, GI:32528299).

cIAP1 is a member of the IAP family of apoptosis inhibitors (Rothe et al., Cell, 83:

1243-1252, 1995; Liston et al., Nature, 379:349-353, 1996). Table 1 provides an amino

25   acid sequence of cIAP1 (also available in GenBank under accession number NP_001157.1,

GI:4502141).

cIAP2 is a member of the IAP family of apoptosis inhibitors (Liston et al., Nature,

379:349-353, 1996). Table 1 provides an amino acid sequence of cIAP2 (also available in

GenBank under accession number NP_031490.1, GI:6680696).

30   NAIP is a member of the IAP family of apoptosis inhibitors (Roy et al., Cell,

80:167-178, 1995). Table 1 provides an amino acid sequence of NAIP (also available in

GenBank under accession number NP_004527.2, GI:119393878). An alternatively spliced

isoform of NAIP is available in GenBank under accession number NP_075043.1,

GI:119393876.

35   Survivin is a member of the IAP family of apoptosis inhibitors (Li et al., Nature,

396:580-584, 1998). Table 1 provides an amino acid sequence of NAIP (also available in

GenBank under accession number NP_001159.2, GI:59859878). Alternatively spliced

isoforms of survivin have been identified (see Wheatley et al., Int. Rev. Cytol., 247:35-88,

15

5   2005; Noton et al., J. Biol. Chem., 281:1286-95, 2006; and Taubert et al., Oncogene,
     24:5258-61, 2005). Sequences of exemplary alternative isoforms are available in
     GenBank under accession numbers NP_001012270.1, GI:59859880, and
     NP_001012271.1, GI:59859882. Exemplary T cell epitopes of survivin include Sur20–28
     (STFKNWPFL; SEQ ID NO:95), Sur96–104 (LTLGEFLKL; SEQ ID NO:96), Sur133–

10   141 (RAIEQLAAM; SEQ ID NO:97), and Sur126–135 (ETAKKVRRAI; SEQ ID NO:98)
     (Bachinsky et al., Cancer Immun., 5:6, 2005). Other exemplary T cell epitopes of survivin
     include Sur92-101 (QFEELTLGEF; SEQ ID NO:99), Sur54-62 (LAQCFFCFK; SEQ ID
     NO:100), Sur112-120 (KIAKETNNK; SEQ ID NO:101), Sur53-62 (DLAQCFFCFK; SEQ
     ID NO:102), Sur112-121 (KIAKETNNKK; SEQ ID NO:103), Sur18-28

15   (RISTFKNWPFL; SEQ ID NO:104), Sur86-96 (FLSVKKQFEEL; SEQ ID NO:105), and
     the modified peptides Sur92T2 (QTEELTLGEF; SEQ ID NO:106), Sur93T2
     (FTELTLGEF; SEQ ID NO:107), Sur93S2 (FSELTLGEF; SEQ ID NO:108), Sur38Y9
     (MAEAGFIHY; SEQ ID NO:109), Sur47Y10 (PTENEPDLAY; SEQ ID NO:110),
     Sur5K9 (TLPPAWQPK; SEQ ID NO:111), Sur54L2 (LLQCFFCFK; SEQ ID NO:112),

20   and Sur18K10 (RISTFKNWPK; SEQ ID NO:113) (Reker et al., Cancer Biol. Ther., 3:173-
     179, 2004). Other exemplary T cell epitopes of survivin include ELTLGEFLKL (SEQ ID
     NO:114) and TLPPAWQPFL (SEQ ID NO:115) (Schmitz et al., Cancer Res. 60:4845-
     4849, 2000). Additional survivin epitopes are described in Siegel et al., Br. J. Haematol.,
     122:911-914, 2003.

25   **Table 1. Sequences of Human Antigens**

Antigen	Amino acid sequence
CD133	MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVHI FLYVVQPRDFPEDTLRKFLQKAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLFIIL MPLVGYFFCMCRCCNKCGGEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVANHQV RTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFTDLNSINSVLGGGILDR LRPNIIPVLDEIKSMATAIKETKEALENMNSTLKSLHQQSTQLSSSLTSVKTSLRSSLNDP LCLVHPSSETCNSIRLSLSQLNSNPELRQLPPVDAELDNVNNVLRTDLDGLVQQGYQSLND IPDRVQRQTTTVVAGIKRVLNSIGSDIDNVTQRLPIQDILSAFSVYVNNTESYIHRNLPTL EEYDSYWWLGGLVICSLLTLIVIFYYLGLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVG LSFLFCWILMIIVVLTFVFGANVEKLICEPYTSKELFRVLDTPYLLNEDWEYYLSGKLFNK SKMKLTFEQVYSDCKKNRGTYGTLHLQNSFNISEHLNINEHTGSISSELESLKVNLNIFLL GAAGRKNLQDFAACGIDRMNYDSYLAQTGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLK RDAQTIKTIHQQRVLPIEQSLSTLYQSVKILQRTGNGLLERVTRILASLDFAQNFITNNTS SVIIEETKKYGRTIIGYFEHYLQWIEFSISEKVASCKPVATALDTAVDVFLCSYIIDPLNL FWFGIGKATVFLLPALIFAVKLAKYYRRMDSEDVYDDVETIPMKNMENGNNGYHKDHVYGI HNPVMTSPSQH (SEQ ID NO:53)
CD90	MNLAISIALLLTVLQVSRGQKVTSLTACLVDQSLRLDCRHENTSSSPIQYEFSLTRETKKH VLFGTVGVPEHTYRSRTNFTSKYNMKVLYLSAFTSKDEGTYTCALHHSGHSPPISSQNVTV LRDKLVKCEGISLLAQNTSWLLLLLLSLSLLQATDFMSL (SEQ ID NO:54)

Antigen	Amino acid sequence
CD44	MDKFWWHAAWGLCLVPLSLAQIDLNITCRFAGVFHVEKNGRYSISRTEAADLCKAFNSTLP TMAQMEKALSIGFETCRYGFIEGHVVIPRIHPNSICAANNTGVYILTSNTSQYDTYCFNAS APPEEDCTSVTDLPNAFDGPITITIVNRDGTRYVQKGEYRTNPEDIYPSNPTDDDVSSGSS SERSSTSGGYIFYTFSTVHPIPDEDSPWITDSTDRIPATTLMSTSATATETATKRQETWDW FSWLFLPSESKNHLHTTTQMAGTSSNTISAGWEPNEENEDERDRHLSFSGSGIDDDEDFIS STISTTPRAFDHTKQNQDWTQWNPSHSNPEVLLQTTTRMTDVDRNGTTAYEGNWNPEAHPP LIHHEHHEEEETPHSTSTIQATPSSTTEETATQKEQWFGNRWHEGYRQTPKEDSHSTTGTA AASAHTSHPMQGRTTPSPEDSSWTDFFNPISHPMGRGHQAGRRMDMDSSHSITLQPTANPN TGLVEDLDRTGPLSMTTQQSNSQSFSTSHEGLEEDKDHPTTSTLTSSNRNDVTGGRRDPNH SEGSTTLLEGYTSHYPHTKESRTFIPVTSAKTGSFGVTAVTVGDSNSNVNRSLSGDQDTFH PSGGSHTTHGSESDGHSHGSQEGGANTTSGPIRTPQIPEWLIILASLLALALILAVCIAVN SRRRCGQKKKLVINSGNGAVEDRKPSGLNGEASKSQEMVHLVNKESSETPDQFMTADETRN LQNVDMKIGV (SEQ ID NO:55)
CXCR4	MSIPLPLLQIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTGIVGNG LVILVMGYQKKLRSMTDKYRLHLSVADLLFVITLPFWAVDAVANWYFGNFLCKAVHVIYTV NLYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSE ADDRYICDRFYPNDLWVVVFQFQHIMVGLILPGIVILSCYCIIISKLSHSKGHQKRKALKT TVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTVHKWISITEALAFFHCCLNPIL YAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS (SEQ ID NO:56)
Nestin	MEGCMGEESFQMWELNRRLEAYLARVKALEEQNELLSAELGGLRAQSADTSWRAHADDELA ALRALVDQRWREKHAAEVARDNLAEELEGVAGRCQQLRLARERTTEEVARNRRAVEAEKCA RAWLSSQVAELERELEALRVAHEEERVGLNAQAACAPRCPAPPRGPPAPAPEVEELARRLG EAWRGAVRGYQERVAHMETSLGQARERLGRAVQGAREGRLELQQLQAERGGLLERRAALEQ RLEGRWQERLRATEKFQLAVEALEQEKQGLQSQIAQVLEGRQQLAHLKMSLSLEVATYRTL LEAENSRLQTPGGGSKTSLSFQDPKLELQFPRTPEGRRLGSLLPVLSPTSLPSPLPATLET PVPAFLKNQEFLQARTPTLASTPIPPTPQAPSPAVDAEIRAQDAPLSLLQTQGGRKQAPEP LRAEARVAIPASVLPGPEEPGGQRQEASTGQSPEDHASLAPPLSPDHSSLEAKDGESGGSR VFSICRGEGEGQIWGLVEKETAIEGKVVSSLQQEIWEEEDLNRKEIQDSQVPLEKETLKSL GEEIQESLKTLENQSHETLERENQECPRSLEEDLETLKSLEKENKELLKDVEVVRPLEKEA VGQLKPTGKEDTQTLQSLQKENQELMKSLEGNLETFLPPGTENQELVSSLQENLESLTALE KENQEPLRSPEVGDEEALRPLTKENQEPLRSLEDENKEAFRSLEKENQEPLKTLEEEDQSI VRPLETENHKSLRSLEEQDQETLRTLEKETQQRRRSLGEQDQMTLRPPEKVDLEPLKSLDQ EIARPLENENQEFLKSLKEESVEAVKSLETEILESLKSAGQENLETLKSPETQAPLWTPEE INQGAMNPLEKEIQEPLESVEVNQETFRLLEEENQESLRSLGAWNLENLRSPEEVDKESQR NLEEEENLGKGEYQESLRSLEEEGQELPQSADVQRWEDTVEKDQELAQESPPGMAGVENED EAELNLREQDGFTGKEEVVEQGELNATEEVWIPGEGHPESPEPKEQRGLVEGASVKGGAEG LQDPEGQSQQVGAPGLQAPQGLPEAIEPLVEDDVAPGGDQASPEVMLGSEPAMGESAAGAE PGPGQGVGGLGDPGHLTREEVMEPPLEEESLEAKRVQGLEGPRKDLEEAGGLGTEFSELPG KSRDPWEPPREGREESEAEAPRGAEEAFPAETLGHTGSDAPSPWPLGSEEAEEDVPPVLVS PSPTYTPILEDAPGPQPQAEGSQEASWGVQGRAEALGKVESEQEELGSGEIPEGPQEEGEE SREESEEDELGETLPDSTPLGFYLRSPTSPRWDPTGEQRPPPQGETGKEGWDPAVLASEGL EAPPSEKEEGEEGEEECGRDSDLSEEFEDLGTEAPFLPGVPGEVAEPLGQVPQLLLDPAAW DRDGESDGFADEEESGEEGEEDQEEGREPGAGRWGPGSSVGSLQALSSSQRGEFLESDSVS VSVPWDDSLRGAVAGAPKTALETESQDSAEPSGSEEESDPVSLEREDKVPGPLEIPSGMED AGPGADIIGVNGQGPNLEGKSQHVNGGVMNGLEQSEEVGQGMPLVSEGDRGSPFQEEEGSA LKTSWAGAPVHLGQGQFLKFTQREGDRESWSSGED (SEQ ID NO:57)

Antigen	Amino acid sequence
Msi1	METDAPQPGLASPDSPHDPCKMFIGGLSWQTTQEGLREYFGQFGEVKECLVMRDPLTKRSR GFGFVTFMDQAGVDKVLAQSRHELDSKTIDPKVAFPRRAQPKMVTRTKKIFVGGLSVNTTV EDVKQYFEQFGKVDDAMLMFDKTTNRHRGFGFVTFESEDIVEKVCEIHFHEINNKMVECKK AQPKEVMSPTGSARGRSRVMPYGMDAFMLGIGMLGYPGFQATTYASRSYTGLAPGYTYQFP EFRVERTPLPSAPVLPELTAIPLTAYGPMAAAAAAAVVRGTGSHPWTMAPPPGSTPSRTG GFLGTTSPGPMAELYGAANQDSGVSSYISAASPAPSTGFGHSLGGPLIATAFTNGYH (SEQ ID NO:58)
MELK	MKDYDELLKYYELHETIGTGGFAKVKLACHILTGEMVAIKIMDKNTLGSDLPRIKTEIEAL KNLRHQHICQLYHVLETANKIFMVLEYCPGGELFDYIISQDRLSEEETRVVFRQIVSAVAY VHSQGYAHRDLKPENLLFDEYHKLKLIDFGLCAKPKGNKDYHLQTCCGSLAYAAPELIQGK SYLGSEADVWSMGILLYVLMCGFLPFDDDNVMALYKKIMRGKYDVPKWLSPSSILLLQQML QVDPKKRISMKNLLNHPWIMQDYNYPVEWQSKNPFIHLDDDCVTELSVHHRNNRQTMEDLI SLWQYDHLTATYLLLLAKKARGKPVRLRLSSFSCGQASATPFTDIKSNNWSLEDVTASDKN YVAGLIDYDWCEDDLSTGAATPRTSQFTKYWTESNGVESKSLTPALCRTPANKLKNKENVY TPKSAVKNEEYFMFPEPKTPVNKNQHKREILTTPNRYTTPSKARNQCLKETPIKIPVNSTG TDKLMTGVISPERRCRSVELDLNQAHMEETPKRKGAKVFGSLERGLDKVITVLTRSKRKGS ARDGPRRLKLHYNVTTTRLVNPDQLLNEIMSILPKKHVDFVQKGYTLKCQTQSDFGKVTMQ FELEVCQLQKPDVVGIRRQRLKGDAWVYKRLVEDILSSCKV (SEQ ID NO:59)
GLI1	MFNSMTPPPISSYGEPCCLRPLPSQGAPSVGTEGLSGPPFCHQANLMSGPHSYGPARETNS CTEGPLFSSPRSAVKLTKKRALSISPLSDASLDLQTVIRTSPSSLVAFINSRCTSPGGSYG HLSIGTMSPSLGFPAQMNHQKGPSPSFGVQPCGPHDSARGGMIPHPQSRGPFPTCQLKSEL DMLVGKCREEPLEGDMSSPNSTGIQDPLLGMLDGREDLEREEKREPESVYETDCRWDGCSQ EFDSQEQLVHHINSEHIHGERKEFVCHWGGCSRELRPFKAQYMLVVHMRRHTGEKPHKCTF EGCRKSYSRLENLKTHLRSHTGEKPYMCEHEGCSKAFSNASDRAKHQNRTHSNEKPYVCKL PGCTKRYTDPSSLRKHVKTVHGPDAHVTKRHRGDGPLPRAPSISTVEPKREREGGPIREES RLTVPEGAMKPQPSPGAQSSCSSDHSPAGSAANTDSGVEMTGNAGGSTEDLSSLDEGPCIA GTGLSTLRRLENLRLDQLHQLRPIGTRGLKLPSLSHTGTTVSRRVGPPVSLERRSSSSSSI SSAYTVSRRSSLASPFPPPGSPPENGASSLPGLMPAQHYLLRARYASARGGGTSPTAASSLD RIGGLPMPPWRSRAEYPGYNPNAGVTRRASDPAQAADRPAPARVQRFKSLGCVHTPPTVAG GGQNFDPYLPTSVYSPQPPSITENAAMDARGLQEEPEVGTSMVGSGLNPYMDFPPTDTLGY GGPEGAAAEPYGARGPGSLPLGPGPPTNYGPNPCPQQASYPDPTQETWGEFPSHSGLYPGP KALGGTYSQCPRLEHYGQVQVKPEQGCPVGSDSTGLAPCLNAHPSEGPPHPQPLFSHYPQP SPPQYLQSGPYTQPPPPDYLPSEPRPCLDFDSPTHSTGQLKAQLVCNYVQSQQELLWEGGGR EDAPAQEPSYQSPKFLGGSQVSPSRAKAPVNTYGPGFGPNLPNHKSGSYPTPSPCHENFVV GANRASHRAAAPPRLLPPLPTCYGPLKVGGTNPSCGHPEVGRLGGGPALYPPPEGQVCNPL DSLDLDNTQLDFVAILDEPQGLSPPPSHDQRGSSGHTPPPSGPPNMAVGNMSVLLRSLPGE TEFLNSSA (SEQ ID NO:60)

Antigen	Amino acid sequence
PTCH1	MASAGNAAEPQDRGGGGSGCIGAPGRPAGGGRRRRTGGLRRAAAPDRDYLHRPSYCDAAFA LEQISKGKATGRKAPLWLRAKFQRLLFKLGCYIQKNCGKFLVVGLLIFGAFAVGLKAANLE TNVEELWVEVGGRVSRELNYTRQKIGEEAMFNPQLMIQTPKEEGANVLTTEALLQHLDSAL QASRVHVYMYNRQWKLEHLCYKSGELITETGYMDQIIEYLYPCLIITPLDCFWEGAKLQSG TAYLLGKPPLRWTNFDPLEFLEELKKINYQVDSWEEMLNKAEVGHGYMDRPCLNPADPDCP ATAPNKNSTKPLDMALVLNGGCHGLSRKYMHWQEELIVGGTVKNSTGKLVSAHALQTMFQL MTPKQMYEHFKGYEYVSHINWNEDKAAAILEAWQRTYVEVVHQSVAQNSTQKVLSFTTTTL DDILKSFSDVSVIRVASGYLLMLAYACLTMLRWDCSKSQGAVGLAGVLLVALSVAAGLGLC SLIGISFNAATTQVLPFLALGVGVDDVFLLAHAFSETGQNKRIPFEDRTGECLKRTGASVA LTSISNVTAFFMAALIPIPALRAFSLQAAVVVVFNFAMVLLIFPAILSMDLYRREDRRLDI FCCFTSPCVSRVIQVEPQAYTDTHDNTRYSPPPPYSSHSFAHETQITMQSTVQLRTEYDPH THVYYTTAEPRSEISVQPVTVTQDTLSCQSPESTSSTRDLLSQFSDSSLHCLEPPCTKWTL SSFAEKHYAPFLLKPKAKVVVIFLFLGLLGVSLYGTTRVRDGLDLTDIVPRETREYDFIAA QFKYFSFYNMYIVTQKADYPNIQHLLYDLHRSFSNVKYVMLEENKQLPKMWLHYFRDWLQG LQDAFDSDWETGKIMPNNYKNGSDDGVLAYKLLVQTGSRDKPIDISQLTKQRLVDADGIIN PSAFYIYLTAWVSNDPVAYAASQANIRPHRPEWVHDKADYMPETRLRIPAAEPIEYAQFPF YLNGLRDTSDFVEAIEKVRTICSNYTSLGLSSYPNGYPFLFWEQYIGLRHWLLLFISVVLA CTFLVCAVFLLNPWTAGIIVMVLALMTVELFGMMGLIGIKLSAVPVVILIASVGIGVEFTV HVALAFLTAIGDKNRRAVLALEHMFAPVLDGAVSTLLGVLMLAGSEFDFIVRYFFAVLAIL TILGVLNGLVLLPVLLSFFGPYPEVSPANGLNRLPTPSPEPPPSVVRFAMPPGHTHSGSDS SDSEYSSQTTVSGLSEELRHYEAQQGAGGPAHQVIVEATENPVFAHSTVVHPESRHHPPSN PRQQPHLDSGSLPPGRQGQQPRRDPPREGLWPPPYRPRRDAFEISTEGHSGPSNRARWGPR GARSHNPRNPASTAMGSSVPGYCQPITTVTASASVTVAVHPPPVPGPGRNPRGGLCPGYPE TDHGLFEDPHVPFHVRCERRDSKVEVIELQDVECEERPRGSSSN (SEQ ID NO:61)
Bmi-1	MHRTTRIKITELNPHLMCVLCGGYFIDATTIIECLHSFCKTCIVRYLETSKYCPICDVQVH KTRPLLNIRSDKTLQDIVYKLVPGLFKNEMKRRRDFYAAHPSADAANGSNEDRGEVADEDK RIITDDEIISLSIEFFDQNRLDRKVNKDKEKSKEEVNDKRYLRCPAAMTVMHLRKFLRSKM DIPNTFQIDVMYEEEPLKDYYTLMDIAYIYTWRRNGPLPLKYRVRPTCKRMKISHQRDGLT NAGELESDSGSDKANSPAGGIPSTSSCLPSPSTPVQSPHPQFPHISSTMNGTSNSPSGNHQ SSFANRPRKSSVNGSSATSSG (SEQ ID NO:62)
PSP	MVSHSELRKLFYSADAVCFDVDSTVIREEGIDELAKICGVEDAVSEMTRRAMGGAVPFKAA LTERLALIQPSREQVQRLIAEQPPHLTPGIRELVSRLQERNVQVFLISGGFRSIVEHVASK LNIPATNVFANRLKFYFNGEYAGFDETQPTAESGGKGKVIKLLKEKFHFKKIIMIGDGATD MEACPPADAFIGFGGNVIRQQVKDNAKWYITDFVELLGELEE (SEQ ID NO:63)
Snail	MPRSFLVRKPSDPNRKPNYSELQDSNPEFTFQQPYDQAHLLAAIPPPEILNPTASLPMLIW DSVLAPQAQPIAWASLRLQESPRVAELTSLSDEDSGKGSQPPSPPSPAPSSFSSTSVSSLE AEAYAAFPGLGQVPKQLAQLSEAKDLQARKAFNCKYCNKEYLSLGALKMHIRSHTLPCVCG TCGKAFSRPWLLQGHVRTHTGEKPFSCPHCSRAFADRSNLRAHLQTHSDVKKYQCQACART FSRMSLLHKHQESGCSGCPR (SEQ ID NO:64)
OCT4	MAGHLASDFAFSPPPGGGGDGPGGPEPGWVDPRTWLSFQGPPGGPGIGPGVGPGSEVWGIP PCPPPYEFCGGMAYCGPQVGVGLVPQGGLETSQPEGEAGVGVESNSDGASPEPCTVTPGAV KLEKEKLEQNPEESQDIKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFGKVFSQTT ICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENRVRGN LENLFLQCPKPTLQQISHIAQQLGLEKDVVRVWFCNRRQKGKRSSSDYAQREDFEAAGSPF SGGPVSFPLAPGPHFGTPGYGSPHFTALYSSVPFPEGEAFPPVSVTTLGSPMHSN (SEQ ID NO:65)

Antigen	Amino acid sequence
BCRP1	MSSSNVEVFIPVSQGNTNGFPATASNDLKAFTEGAVLSFHNICYRVKLKSGFLPCRKPVEK EILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCNSG YVVQDDVVMGTLTVRENLQFSAALRLATTMTNHEKNERINRVIQELGLDKVADSKVGTQFI RGVSGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIFSIHQ PRYSIFKLFDSLTLLASGRLMFHGPAQEALGYFESAGYHCEAYNNPADFFLDIINGDSTAV ALNREEDFKATEIIEPSKQDKPLIEKLAEIYVNSSFYKETKAELHQLSGGEKKKKITVFKE ISYTTSFCHQLRWVSKRSFKNLLGNPQASIAQIIVTVVLGLVIGAIYFGLKNDSTGIQNRA GVLFFLTTNQCFSSVSAVELFVVEKKLFIHEYISGYYRVSSYFLGKLLSDLLPMRMLPSII FTCIVYFMLGLKPKADAFFVMMFTLMMVAYSASSMALAIAAGQSVVSVATLLMTICFVFMM IFSGLLVNLTTIASWLSWLQYFSIPRYGFTALQHNEFLGQNFCPGLNATGNNPCNYATCTG EEYLVKQGIDLSPWGLWKNHVALACMIVIFLTIAYLKLLFLKKYS (SEQ ID NO:66)
MGMT	MDKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPLMQ CTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQLAALAG NPKAARAVGGAMRGNPVPILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGHRLGKPGLGG SSGLAGAWLKGAGATSGSPPAGRN (SEQ ID NO:77)
BCL-2	MAHAGRTGYDNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGAAPAPGIFSSQPGHTPHPAA SRDPVARTSPLQTPAAPGAAAGPALSPVPPVVHLTLRQAGDDFSRRYRRDFAEMSSQLHLT PFTARGRFATVVEELFRDGVNWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEYLNR HLHTWIQDNGGWDAFVELYGPSMRPLFDFSWLSLKTLLSLALVGACITLGAYLGHK (SEQ ID NO:78)
FLIP	MSAEVIHQVEEALDTDEKEMLLFLCRDVAIDVVPPNVRDLLDILRERGKLSVGDLAELLYR VRRFDLLKRILKMDRKAVETHLLRNPHLVSDYRVLMAEIGEDLDKSDVSSLIFLMKDYMGR GKISKEKSFLDLVVELEKLNLVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQSVQGAGTSYR NVLQAAIQKSLKDPSNNFRLHNGRSKEQRLKEQLGAQQEPVKKSIQESEAFLPQSIPEERY KMKSKPLGICLIIDCIGNETELLRDTFTSLGYEVQKFLHLSMHGISQILGQFACMPEHRDY DSFVCVLVSRGGSQSVYGVDQTHSGLPLHHIRRMFMGDSCPYLAGKPKMFFIQNYVVSEGQ LENSSLLEVDGPAMKNVEFKAQKRGLCTVHREADFFWSLCTADMSLLEQSHSSPSLYLQCL SQKLRQERKRPLLDLHIELNGYMYDWNSRVSAKEKYYVWLQHTLRKKLILSYT (SEQ ID NO:79)
BCL-XL	MSQSNRELVVDFLSYKLSQKGYSWSQFSDVEENRTEAPEGTESEMETPSAINGNPSWHLAD SPAVNGATGHSSSLDAREVIPMAAVKQALREAGDEFELRYRRAFSDLTSQLHITPGTAYQS FEQVVNELFRDGVNWGRIVAFFSFGGALCVESVDKEMQVLVSRIAAWMATYLNDHLEPWIQ ENGGWDTFVELYGNNAAAESRKGQERFNRWFLTGMTVAGVVLLGSLFSRK (SEQ ID NO:80)
XIAP	MTFNSFEGSKTCVPADINKEEEFVEEFNRLKTFANFPSGSPVSASTLARAGFLYTGEGDTV RCFSCHAAVDRWQYGDSAVGRHRKVSPNCRFINGFYLENSATQSTNSGIQNGQYKVENYLG SRDHFALDRPSETHADYLLRTGQVVDISDTIYPRNPAMYSEEARLKSFQNWPDYAHLTPRE LASAGLYYTGIGDQVQCFCCGGKLKNWEPCDRAWSEHRRHFPNCFFVLGRNLNIRSESDAV SSDRNFPNSTNLPRNPSMADYEARIFTFGTWIYSVNKEQLARAGFYALGEGDKVKCFHCGG GLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTHSLEECLVRTTEKTPSLTRRI DDTIFQNPMVQEAIRMGFSFKDIKKIMEEKIQISGSNYKSLEVLVADLVNAQKDSMQDESS QTSLQKEISTEEQLRRLQEEKLCKICMDRNIAIVFVPCGHLVTCKQCAEAVDKCPMCYTVI TFKQKIFMS (SEQ ID NO:81)

Antigen	Amino acid sequence
cIAP1	MHKTASQRLFPGPSYQNIKSIMEDSTILSDWTNSNKQKMKYDFSCELYRMSTYSTFPAGVP VSERSLARAGFYYTGVNDKVKCFCCGLMLDNWKLGDSPIQKHKQLYPSCSFIQNLVSASLG STSKNTSPMRNSFAHSLSPTLEHSSLFSGSYSSLSPNPLNSRAVEDISSSRTNPYSYAMST EEARFLTYHMWPLTFLSPSELARAGFYYIGPGDRVACFACGGKLSNWEPKDDAMSEHRRHF PNCPFLENSLETLRFSISNLSMQTHAARMRTFMYWPSSVPVQPEQLASAGFYYVGRNDDVK CFCCDGGLRCWESGDDPWVEHAKWFPRCEFLIRMKGQEFVDEIQGRYPHLLEQLLSTSDTT GEENADPPIIHFGPGESSSEDAVMMNTPVVKSALEMGFNRDLVKQTVQSKILTTGENYKTV NDIVSALLNAEDEKREEEKEKQAEEMASDDLSLIRKNRMALFQQLTCVLPILDNLLKANVI NKQEHDIIKQKTQIPLQARELIDTILVKGNAAANIFKNCLKEIDSTLYKNLFVDKNMKYIP TEDVSGLSLEEQLRRLQEERTCKVCMDKEVSVVFIPCGHLVVCQECAPSLRKCPICRGIIK GTVRTFLS (SEQ ID NO:82)
cIAP2	MVQDSAFLAKLMKSADTFELKYDFSCELYRLSTYSAFPRGVPVSERSLARAGFYYTGANDK VKCFCCGLMLDNWKQGDSPMEKHRKLYPSCNFVQTLNPANSLEASPRPSLPSTAMSTMPLS FASSENTGYFSGSYSSFPSDPVNFRANQDCPALSTSPYHFAMNTEKARLLTYETWPLSFLS PAKLAKAGFYYIGPGDRVACFACDGKLSNWERKDDAMSEHQRHFPSCPFLKDLGQSASRYT VSNLSMQTHAARIRTFSNWPSSALVHSQELASAGFYYTGHSDDVKCFCCDGGLRCWESGDD PWVEHAKWFPRCEYLLRIKGQEFVSQVQAGYPHLLEQLLSTSDSPEDENADAAIVHFGPGE SSEDVVMMSTPVVKAALEMGFSRSLVRQTVQRQILATGENYRTVSDLVIGLLDAEDEMREE QMEQAAEEEESDDLALIRKNKMVLFQHLTCVTPMLYCLLSARAITEQECNAVKQKPHTLQA STLIDTVLAKGNTAATSFRNSLREIDPALYRDIFVQQDIRSLPTDDIAALPMEEQLRKLQE ERMCKVCMDREVSIVFIPCGHLVVCKDCAPSLRKCPICRGTIKGTVRTFLS (SEQ ID NO:83)
NAIP	MATQQKASDERISQFDHNLLPELSALLGLDAVQLAKELEEEEQKERAKMQKGYNSQMRSEA KRLKTFVTYEPYSSWIPQEMAAAGFYFTGVKSGIQCFCCSLILFGAGLTRLPIEDHKRFHP DCGFLLNKDVGNIAKYDIRVKNLKSRLRGGKMRYQEEEARLASFRNWPFYVQGISPCVLSE AGFVFTGKQDTVQCFSCGGCLGNWEEGDDPWKEHAKWFPKCEFLRSKKSSEEITQYIQSYK GFVDITGEHFVNSWVQRELPMASAYCNDSIFAYEELRLDSFKDWPRESAVGVAALAKAGLF YTGIKDIVQCFSCGGCLEKWQEGDDPLDDHTRCFPNCPFLQNMKSSAEVTPDLQSRGELCE LLETTSESNLEDSIAVGPIVPEMAQGEAQWFQEAKNLNEQLRAAYTSASFRHMSLLDISSD LATDHLLGCDLSIASKHISKPVQEPLVLPEVFGNLNSVMCVEGEAGSGKTVLLKKIAFLWA SGCCPLLNRFQLVFYLSLSSTRPDEGLASIICDQLLEKEGSVTEMCVRNIIQQLKNQVLFL LDDYKEICSIPQVIGKLIQKNHLSRTCLLIAVRTNRARDIRRYLETILEIKAFPFYNTVCI LRKLFSHNMTRLRKFMVYFGKNQSLQKIQKTPLFVAAICAHWFQYPFDPSFDDVAVFKSYM ERLSLRNKATAEILKATVSSCGELALKGFFSCCFEFNDDDLAEAGVDEDEDLTMCLMSKFT AQRLRPFYRFLSPAFQEFLAGMRLIELLDSDRQEHQDLGLYHLKQINSPMMTVSAYNNFLN YVSSLPSTKAGPKIVSHLLHLVDNKESLENISENDDYLKHQPEISLQMQLLRGLWQICPQA YFSMVSEHLLVLALKTAYQSNTVAACSPFVLQFLQGRTLTLGALNLQYFFDHPESLSLLRS IHFPIRGNKTSPRAHFSVLETCFDKSQVPTIDQDYASAFEPMNEWERNLAEKEDNVKSYMD MQRRASPDLSTGYWKLSPKQYKIPCLEVDVNDIDVVGQDMLEILMTVFSASQRIELHLNHS RGFIESIRPALELSKASVTKCSISKLELSAAEQELLLTLPSLESLEVSGTIQSQDQIFPNL DKFLCLKELSVDLEGNINVFSVIPEEFPNFHHMEKLLIQISAEYDPSKLVKLIQNSPNLHV FHLKCNFFSDFGSLMTMLVSCKKLTEIKFSDSFFQAVPFVASLPNFISLKILNLEGQQFPD EETSEKFAYILGSLSNLEELILPTGDGIYRVAKLIIQQCQQLHCLRVLSFFKTLNDDSVVE IAKVAISGGFQKLENLKLSINHKITEEGYRNFFQALDNMPNLQELDISRHFTECIKAQATT VKSLSQCVLRLPRLIRLNMLSWLLDADDIALLNVMKERHPQSKYLTILQKWILPFSPIIQK (SEQ ID NO:84)
Survivin	MGAPTLPPAWQPFLKDHRISTFKNWPFLEGCACTPERMAEAGFIHCPTENEPDLAQCFFCF KELEGWEPDDDPIEEHKKHSSGCAFLSVKKQFEELTLGEFLKLDRERAKNKIAKETNNKKK EFEETAEKVRRAIEQLAAMD (SEQ ID NO:85)

5

5   T cell epitopes can be identified by a number of different methods. Naturally
processed MHC epitopes can be identified by mass spectrophotometric analysis of
peptides eluted from antigen-loaded APC (e.g., APC that have taken up antigen, or that
have been engineered to produce the protein intracellularly). After incubation at 37 °C,
cells are lysed in detergent and the MHC protein is purified (e.g., by affinity
10   chromatography). Treatment of the purified MHC with a suitable chemical medium (e.g.,
under acidic conditions) results in the elution of peptides from the MHC. This pool of
peptides is separated and the profile compared with peptides from control APC treated in
the same way. The peaks unique to the protein expressing/fed cells are analyzed (for
example by mass spectrometry) and the peptide fragments identified. This protocol
15   identifies peptides generated from a particular antigen by antigen processing.

        Alternatively, epitopes can be identified by screening a synthetic library of peptides
that overlap and span the length of the antigen in an in vitro assay. For example, peptides
that are 9 amino acids in length and which overlap by 5 amino acids may be used. The
peptides are tested in an antigen presentation system that includes antigen presenting cells
20   and T cells. T cell activation in the presence of APCs presenting the peptide can be
measured (e.g., by measuring T cell proliferation or cytokine production) and compared to
controls, to determine whether a particular epitope is recognized by the T cells.

        T cell epitopes can be predicted in silico, e.g., using the methods described in
Parker et al., J. Immunol., 152:163, 1994 and Rammensee et al., Immunogenet., 50:213-
25   219, 1999.

        Antigenic peptides can be obtained by chemical synthesis using a commercially
available automated peptide synthesizer. Synthetic peptides can be precipitated and
further purified, for example by high performance liquid chromatography (HPLC).
Alternatively, isolated peptides can be obtained by purification and/or recombinant
30   methods using host cell and vector expression systems.

Preparation of Antigen Presenting Cells

        Antigen presenting cells (APC), such as DCs, suitable for administration to
subjects (e.g., glioma patients) can be isolated or obtained from any tissue in which such
35   cells are found, or may be otherwise cultured and provided using standard techniques.
Methods of preparing antigen presenting cells are well-known to those of skill in the art.
Mature dendritic cells are typically identified as having the following cell surface marker

5   phenotype: MAC3$^-$, CD80$^+$, CD86$^+$, CD40^{low}, CD54$^+$, MHC Class I and MHC Class II,
     and are capable of FITC-dextran uptake.

          APCs (e.g., DCs) can be found, by way of example, in the bone marrow or PBMCs
     of a mammal, in the spleen of a mammal or in the skin of a mammal (i.e., Langerhan's
     cells, which possess certain qualities similar to that of DC, may be found in the skin). For

10   instance, bone marrow can be harvested from a mammal and cultured in a medium that
     promotes the growth of DCs. GM-CSF, IL-4 and/or other cytokines (e.g., TNF-α), growth
     factors and supplements may be included in this medium.

          After a suitable amount of time in culture in medium containing appropriate
     cytokines (e.g., time suitable to expand and differentiate the DCs into mature DCs, e.g., 2,

15   4, 6, 8, 10, 12, or 15 days), clusters of DCs are cultured in the presence of a sufficient
     number of antigens of interest (e.g., in the presence of cancer stem cell lysate, acid eluted
     peptides of cancer stem cells, peptides of CD133, CD90, CD44, CXCR4, Nestin, Musashi-
     1 (Msi1), maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1,
     phosphoserine phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL,

20   XIAP, cIAP1, cIAP2, NAIP, or survivin, or a combination of two or more of the above
     antigens) and harvested for use in a cancer vaccine. For example, peptide antigens can be
     added to the culture medium at a concentration of about 1.0 to 50, e.g., 5, 10, 15, 20, or 30
     μg/ml (per antigen).

          Alternately, or in combination, antigens can be transgenically expressed in DCs,

25   e.g., by transfection of nucleic acids encoding one or more of the antigens or portions of
     antigens.

          In one exemplary method, APCs are isolated from a subject (e.g., a human)
     according to the following exemplary procedure. Mononuclear cells are isolated from
     blood using leukapheresis (e.g., using a COBE Spectra Apheresis System). The

30   mononuclear cells are allowed to become adherent by incubation in tissue culture flasks
     for 2 hours at 37 °C. Nonadherent cells are removed by washing. Adherent cells are
     cultured in medium supplemented with granulocyte macrophage colony stimulating factor
     (GM-CSF) and interleukin-4 (IL-4) for five days. On day five, TNF-α is added to the
     culture medium for another 3-4 days. On day 8 or 9, cells are harvested and washed, and

35   incubated with peptide antigens for 16-20 hours on a tissue rotator. Peptide antigens are
     added to the cultures at a concentration of ~ 10 μg/ml (per antigen).

5   Various other methods can be used to isolate the APCs, as would be recognized by one of skill in the art. DCs occur in low numbers in all tissues in which they reside, making isolation and enrichment of DCs a requirement. Any of a number of procedures entailing repetitive density gradient separation, fluorescence activated cell sorting techniques, positive selection, negative selection or a combination thereof are routinely
10   used to obtain enriched populations or isolated DCs. Guidance on such methods for isolating DCs can be found in O'Doherty et al., J. Exp. Med., 178:1067-78, 1993; Young and Steinman, J. Exp. Med., 171:1315-32, 1990; Freudenthal and Steinman, Proc. Nat. Acad. Sci. USA, 57:7698-7702, 1990; Macatonia et al., 67:285-289, 1989; Markowicz and Engleman, J. Clin. Invest., 85:955-961, 1990; Mehta-Damani et al., J. Immunol., 153:996-
15   1003, 1994; and Thomas et al., J. Immunol., 151:6840-6852, 1993. One method for isolating DCs from human peripheral blood is described in U.S. Patent No. 5,643,786. Methods of producing DCs from embryonic stem cells are described in U.S. Patent No. 7,247,480.

Administration of Cancer Vaccines

20   The APC-based cancer vaccine may be delivered to a recipient by any suitable delivery route, which can include injection, infusion, inoculation, direct surgical delivery, or any combination thereof. In some embodiments, the cancer vaccine is administered to a human in the deltoid region or axillary region. In some embodiments, the vaccine is administered to a subject locally to the site of a tumor, within the tumor, or to an area from
25   which a tumor has been surgically resected.

        An appropriate carrier for administering the cells may be selected by one of skill in the art by routine techniques. For example, the pharmaceutical carrier can be a buffered saline solution, e.g., cell culture media.

        The quantity of APC appropriate for administration to a patient as a cancer vaccine
30   to effect the methods of the present invention and the most convenient route of such administration may be based upon a variety of factors, as may the formulation of the vaccine itself. Some of these factors include the physical characteristics of the patient (e.g., age, weight, and sex), the physical characteristics of the tumor (e.g., location, size, rate of growth, and accessibility), and the extent to which other therapeutic methodologies
35   (e.g., chemotherapy, and beam radiation therapy) are being implemented in connection with an overall treatment regimen. Notwithstanding the variety of factors one should consider in implementing the methods of the present invention to treat a disease condition, a mammal can be administered with from about 10^5 to about 10^9 APC (e.g., 10^7 APC) in

5   from about 0.05 mL to about 5 mL solution (e.g., saline) in a single administration.
Additional administrations can be carried out, depending upon the above-described and
other factors, such as the severity of tumor pathology. In one embodiment, from about one
to about five administrations of about 10^6 APC is performed at two-week intervals.

         DC vaccination can be accompanied by other treatments. For example, a patient
10   receiving DC vaccination may also be receiving chemotherapy, radiation, and/or surgical
therapy concurrently. Methods of treating cancer using DC vaccination in conjunction
with chemotherapy are described in Wheeler et al., U.S. Pat. Pub. No. 2007/0020297. In
some embodiments, a patient receiving DC vaccination has already received
chemotherapy, radiation, and/or surgical treatment for the cancer. In one embodiment, a
15   patient receiving DC vaccination is treated with a COX-2 inhibitor, as described in Yu and
Akasaki, WO 2005/037995.

Pharmaceutical Compositions

         In various embodiments, the present invention provides pharmaceutical
compositions including a pharmaceutically acceptable excipient along with a
20   therapeutically effective amount of the inventive vaccine comprising dendritic cells pulsed
with cancer stem cell antigens as described herein. "Pharmaceutically acceptable
excipient" means an excipient that is useful in preparing a pharmaceutical composition that
is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for
veterinary use as well as for human pharmaceutical use. Such excipients may be solid,
25   liquid, semisolid, or, in the case of an aerosol composition, gaseous.

         In various embodiments, the pharmaceutical compositions according to the
invention may be formulated for delivery via any route of administration. "Route of
administration" may refer to any administration pathway known in the art, including but
not limited to aerosol, nasal, transmucosal, transdermal, or parenteral. "Parenteral" refers
30   to a route of administration that is generally associated with injection, including
intraorbital, infusion, intraarterial, intracapsular, intracardiac, intradermal, intramuscular,
intraperitoneal, intrapulmonary, intraspinal, intrasternal, intrathecal, intrauterine,
intravenous, subarachnoid, subcapsular, subcutaneous, transmucosal, or transtracheal. Via
the parenteral route, the compositions may be in the form of solutions or suspensions for
35   infusion or for injection, or as lyophilized powders.

         The pharmaceutical compositions according to the invention can also contain any
pharmaceutically acceptable carrier. "Pharmaceutically acceptable carrier" as used herein
refers to a pharmaceutically acceptable material, composition, or vehicle that is involved in

                                                    25

5   carrying or transporting a compound of interest from one tissue, organ, or portion of the

body to another tissue, organ, or portion of the body. For example, the carrier may be a

liquid or solid filler, diluent, excipient, solvent, or encapsulating material, or a combination

thereof. Each component of the carrier must be "pharmaceutically acceptable" in that it

must be compatible with the other ingredients of the formulation. It must also be suitable

10   for use in contact with any tissues or organs with which it may come in contact, meaning

that it must not carry a risk of toxicity, irritation, allergic response, immunogenicity, or any

other complication that excessively outweighs its therapeutic benefits.

The pharmaceutical compositions according to the invention may be delivered in a

therapeutically effective amount. The precise therapeutically effective amount is that

15   amount of the composition that will yield the most effective results in terms of efficacy of

treatment in a given subject. This amount will vary depending upon a variety of factors,

including but not limited to the characteristics of the therapeutic compound (including

activity, pharmacokinetics, pharmacodynamics, and bioavailability), the physiological

condition of the subject (including age, sex, disease type and stage, general physical

20   condition, responsiveness to a given dosage, and type of medication), the nature of the

pharmaceutically acceptable carrier or carriers in the formulation, and the route of

administration. One skilled in the clinical and pharmacological arts will be able to

determine a therapeutically effective amount through routine experimentation, for instance,

by monitoring a subject's response to administration of a compound and adjusting the

25   dosage accordingly. For additional guidance, see Remington: The Science and Practice of

Pharmacy (Gennaro ed. 20th edition, Williams & Wilkins PA, USA) (2000).

Kits

Kits to treat cancer are also contemplated. The kits are useful for practicing the

30   inventive method of treating cancer with a vaccine comprising dendritic cells pulsed with

cancer stem cell antigens as described herein. The kit is an assemblage of materials or

components, including at least one of the inventive compositions. Thus, in some

embodiments the kit contains a composition including a vaccine comprising dendritic cells

pulsed with cancer stem cell antigens as described herein.

35   The exact nature of the components configured in the inventive kit depends on its

intended purpose. For example, some embodiments are configured for the purpose of

treating a particular cancer. In one embodiment, the kit is configured for the purpose of

treating brain tumors. In one particular embodiment, the brain tumor is a glioma. In

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5 another embodiment, the brain tumor is GBM. In one embodiment, the kit is configured particularly for the purpose of treating mammalian subjects. In another embodiment, the kit is configured particularly for the purpose of treating human subjects. In further embodiments, the kit is configured for veterinary applications, treating subjects such as, but not limited to, farm animals, domestic animals, and laboratory animals.

10 Instructions for use may be included in the kit. "Instructions for use" typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as induction of an immune response against a tumor, to treat a cancer. For example, the instructions may comprise instructions to administer a vaccine comprising dendritic cells pulsed with cancer stem cell

15 antigens to the patient.

Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, or other useful paraphernalia as will be readily recognized by those of skill in the art.

20 The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging material(s). As employed herein, the phrase

25 "packaging material" refers to one or more physical structures used to house the contents of the kit, such as inventive compositions and the like. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. The packaging materials employed in the kit are those customarily utilized in cancer treatments or in vaccinations. As used herein, the term "package" refers to a

30 suitable solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding the individual kit components. Thus, for example, a package can be a glass vial used to contain suitable quantities of an inventive composition containing for example, a vaccine comprising dendritic cells pulsed with cancer stem cell antigens as described herein. The packaging material generally has an external label which indicates the

35 contents and/or purpose of the kit and/or its components.

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EXAMPLES

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

Example 1. The 9L Gliosarcoma Cell Line Contains Self-Renewing Cells

To determine if a population of self-renewing stem cells exist within the phenotypically heterogeneous 9L gliosarcoma tumor, the cells were grown as monolayers in the presence of 10% FBS and subsequently grew them in serum free media containing mitogens. A schematic diagram of culturing of tumor stem cells is presented in Fig. 2. 9L gliosarcomas were resuspended in Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal bovine serum (FBS) and plated at a density of 1×10^6 live cells per 75 cm^2 flask. The cells attached and grew as monolayers and were passaged upon confluency. Spheres were derived by placing the 9L gliosarcomas cells grown as monolayers into a defined serum-free NSC medium (Reynolds et al., J. Neurosci., 12: 4565-74, 1992; Reynolds et al., Science, 255:1707-10, 1992) consisting of Dulbecco's modified Eagle's medium/F-12 medium supplemented with 20 ng/mL of both epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ) and basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ). Cells were fed every 2 days by adding fresh NSC media supplemented with growth factors. After primary spheres formed and reached 10-200 cells per sphere, the cells were harvested, dissociated into single cells using trypsin and EDTA (GIBCO BRL) and mechanical pipetting, strained through a cell strainer, and plated at a clonal density of 1,000 cells/mL in neurosphere-conditioned medium to generate clonally derived subspheres (Geschwind et al., Neuron, 29:325-339, 2001; Groszer et al., Science, 294: 2186-89, 2001). The cells were fed every 2 days by adding fresh NSC media supplemented with mitogens. Cells and subsequent spheres were observed daily for 18 days, and passaged into fresh media. Subspheres ranging from approximately 15 cells to 40 cells were evident after 18 days and displayed the self-renewing and proliferative capacity of the 9L spheres.

Subtle differences were observed in surface adhesion properties between the two cell populations when using different coating solutions. It was found that poly-L-lysine proved less effective in binding to neurospheres than did laminin. Even after 2 days, only

5   a small portion of the cells adhered (~ 5%). However, when neurospheres were allowed to adhere to chamber slides coated with laminin, nearly all the cells (~ 70%) adhered within the first 3 hours. As a consequence, poly-L-lysine was not used as an adhesive substrate in further studies. A similar trend was observed when comparing adhesion of monolayers to chamber slides coated with either poly-L-lysine or laminin—cellular morphology was

10   more apparent in monolayers grown on laminin coated chamber slides within a 6 hour time period. Furthermore, a distinct morphological characteristic was apparent in the differentiated neurospheres, where arms of differentiated cells originating from one neurosphere homed to arms formed by another nearby neurosphere (FIG. 8A). These examples of extracellular adhesion and cellular homing can be used to distinguish cancer

15   stem-like cells (CSLCs) from non-CSLCs within a tumor.

Example 2. 9L Neurospheres Express NSC Markers and Can Generate Both Neuronal and Glial Cells in Culture

        To determine the expression of markers for stem cells, neurospheres were

20   immunostained for NSC markers nestin and Sox2. Neurospheres were also stained for the lineage markers for astrocytes, GFAP, neurons, beta-tubulin III and MAP2, and oligodendrocytes, myelin/oligodendrocyte. Cells in the outer region of the neurosphere labeled for nestin, while cells throughout the neurosphere were labeled for Sox2. A large number of cells within the tumor spheres were also found to be positive for the lineage

25   marker GFAP, while relatively few cells expressed the neuronal lineage markers β-tubulin III , MAP2, and myelin/oligodendrocyte.

        To test whether spheres have multipotent capabilities and produce progenies of different lineages, spheres were seeded into chamber slides (Lab-TekII, Nalge Nunc International) for differentiation assay. The cells were grown for 14 days in medium

30   devoid of growth factors bFGF and EGF but permissive for differentiation, and processed for immunocytochemistry as described below. The medium included Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal bovine serum (FBS).

        To examine the expression of NSC markers and lineage markers, immuncyto- and immunohistochemical staining was performed. For staining of differentiated spheres,

35   spheres and 9L monolayers, cells growing in chamber slides were fixed with 4% paraformaldehyde for 15 minutes at 4 °C, treated with 5% NHS (normal horse serum)/0.1% Triton-X, and then stained with the following antibodies: rabbit anti-nestin (1:200, Chemicon), rabbit anti-Sox2 (1:1,000, Chemicon), rabbit anti-MAP2 (1:1,000,

5   Sigma), mouse anti-β-tubulin III (1:200, Chemicon), rabbit anti-GFAP (1:1000,
     Chemicon), mouse anti-myelin/oligodendrocyte (1:1,000, Chemicon). The primary
     antibodies were detected with Cy3 or FITC-conjugated anti-mouse or anti-rabbit IgG
     antibody (1:200, Jackson Immuno Research). The cells were counterstained with 4',6-
     diamidino-2-phenylindole (DAPI; Vector Laboratories) to identify all nuclei. The stained

10   sections were examined and photographed using a QED cell scanner program and Nikon
     Eclipse TE2000-E microscope, and analyzed using Image J (NIH). For immunostaining of
     spheres, the spheres where allowed to adhere to precoated (with laminin) chamber slides
     for 3 hours before fixation, while monolayers were allowed to adhere overnight in non-
     fixed chamber slides.

15   Cells stained positive for the NSC markers nestin and Sox2, as well as the lineage
     markers for astrocytes, GFAP, neurons, MAP2, and oligodendrocytes,
     myelin/oligodendrocyte. However, no cells were labeled with the neuronal marker beta-
     tubulin III. FACS analysis showed similar results to those derived via
     immunocytochemistry, which showed differentiated cells labeled for nestin (88%; FIG.

20   3C), Sox2 (24%), GFAP (96%; FIG. 3B), MAP2 (29%; FIG. 3D), and
     myelin/oligodendrocyte (6%; FIG. 3F), but not for β-tubulin III (0%; FIG. 3E). For FACS
     analysis, cells were fixed with 4% paraformaldehyde for 15 minutes at 4 °C, treated with
     5% NHS (normal horse serum)/0.1% Triton™-X, and then stained with the following
     antibodies: rabbit anti-nestin (1:1,000, Chemicon), rabbit anti-Sox2 (1:1,000, Chemicon),

25   rabbit anti-MAP2 (1:1,000, Sigma), mouse anti-β-tubulin III (1:500, Chemicon), rabbit
     anti-GFAP (1:1000, Chemicon), and mouse anti-myelin/oligodendrocyte (1:1,000,
     Chemicon). The primary antibodies were detected with FITC-conjugated anti-mouse or
     anti-rabbit IgG antibody (1:200, Jackson Immuno Research) using 10^4 cells in a
     FACSVantage™ fluorescence activated cell sorter (Becton Dickinson). These results

30   indicate that these cancer stem-like cells (CSLCs), unlike normal stem cells, differentiate
     into aberrant cells that are positive for multiple differentiation markers, notably GFAP and
     MAP2. Such a dual nature is apparent in the majority of cells differentiated from
     neurospheres. Furthermore, many cells still remained highly positive for nestin and Sox2.
     The expression pattern of the differentiated progeny was similar in profile to that of

35   primary cultured tumor cells from which the spheres had originally been isolated and
     predominantly differentiated into GFAP and MAP2 positive cells that recapitulated the
     parental tumor phenotype. Additionally, the level of labeling for the NSC markers nestin
     and Sox2 still remained high, even after 14 days of differentiation. The staining pattern for

5   the monolayer population was similar to neurospheres differentiated for 14 days, except

for the expression of Sox2, which was greater in neurospheres differentiated for 14 days.

These results reveal that 9L spheres are multipotent for the three neural cell types and

differentiate into cells found in the original tumor from which they were obtained.

10   Example 3. The Aggressiveness of 9L Cells in vivo is Reliant on the Neurosphere Cancer
Stem-Like Cells

To determine if 9L cells grown as monolayer or neurospheres differ in their ability

to grow as a tumor after implantation 5,000 cells from both populations of cells were

injected into rats and survival and tumor volume were assayed.

15   Fisher F344 rats 6-8 weeks old (Harlan Sprague-Dawley, Indianapolis, IN) were

anesthetized with i.p. ketamine and xylazine, and stereotactically implanted in the right

striatum (from bregma in mm: anterior-posterior +1 mm; medial-lateral –3 mm; dorsal-

ventral –5 mm) with either isolated 9L sphere cells containing the luciferase gene (5,000

cells) or non-sphere-forming monolayer cells containing the luciferase gene (5,000 cells)

20   in 4µL of 1.2% methylcellulose/PBS (Rehemtulla et al., Neoplasia, 2:491-495, 2000).

Rats were portioned to either the tumor volume group (n = 10) or the survival group (n =

18), with control rats (n = 6) receiving 4µL of 1.2% methylcellulose/PBS only.

Because half of each group consisted of either animals implanted with spheres or

monolayers, tumor aggression could be determined. Animals in the tumor volume group

25   were sacrificed 18 days after tumor implantation. Tumor volume was assessed by using

the formula for an ellipsoid, (length x width x height)/2 (Advani et al., Cancer Res.,

59:2055-58, 1999), with the height and the width of the tumor being approximately equal

because of the well-defined circumference of the tumors generated by the 9L gliosarcoma

(see Sibenaller et al., Neurosurg. Focus, 19:E1, 2005). A significantly (P < 0.02) greater

30   tumor volume was observed in the rats implanted with neurosphere cells as compared to

rats implanted with monolayer cells (FIG. 4A).

Animals in the survival group were followed for survival and euthanized via CO_2

asphyxiation when terminal neurological signs developed (e.g., inability to access food,

water, seizure activity, weakness, and paralysis) or if animals exceeded a survival period

35   of 40 days. Following euthanization, brains were harvested and frozen in 2-methylbutane

(Sigma), cooled to –20 °C, and stored at –80 °C until sectioning. For H&E staining, 20 µm

coronal brain sections described above were mounted on slide and stained with Harris

5   hematoxylin for 2 minutes and then counterstained with alcoholic eosin. Reticulin stains
     were performed on 12 μm coronal brain sections.

            Similar to the results seen in the tumor size groups, rats implanted with
     neurospheres on average had shorter survival times than rats implanted with monolayer
     cells (FIG. 4B). Six rats in the neurosphere group had large tumors that lead to terminal
10   neurological symptoms, whereas 3 showed evidence of small tumors after 40 days of
     survival as seen by H&E staining of brain sections. In contrast, only 4 rats had tumors
     large enough to create terminal neurological symptoms in the monolayer group, whereas 5
     showed evidence of small tumor or engraftment after 40 days of survival as determined by
     H&E staining. Furthermore, the rats in the monolayer group which developed terminal
15   neurological signs of tumor did so at a later time (36 days compared to 29 days in the
     neurosphere group), which was significant when analyzed using the Kaplan-Meier test (P
     < 0.02).

            To determine the establishment of tumor *in vivo*, 150 mg/kg of the luciferase
     substrate D-luciferin (Biosynth, International, Inc., Naperville, IL) was administered i.p. to
20   animals in the survival group, and luciferase scans were taken 15 minutes later. Luciferase
     scans generated at 14 days post implantation demonstrated a greater proportion of animals
     with tumor burden in the neurosphere group compared to the monolayer group, even
     though a higher expression level of luciferase in vitro was observed in monolayer cells as
     compared to neurosphere cells.

25   To determine if the 9L neurospheres could recapitulate the 9L sarcoma in vivo,
     histological analysis of the tumors was performed. The tumor-cell-implanted rat brains
     were cut with a cryostat into 20 μm coronal sections and fixed in 4% paraformaldehyde,
     washed with PBS, and air dried. To characterize the brain tissue by
     immunohistochemistry, free-floating sections were blocked with 5% NHS (normal horse
30   serum) for 30 minutes at room temperature and then stained with the following antibodies:
     rabbit anti-nestin (1:200, Chemicon), rabbit anti-Sox2 (1:1,000, Chemicon), rabbit anti-
     MAP2 (1:1,000, Sigma), mouse anti-β-tubulin III (1:200, Chemicon), rabbit anti-GFAP
     (1:1000, Chemicon), mouse anti-myelin/oligodendrocyte (1:1000; Chemicon). The
     primary antibodies were detected as in Example 1. The cells were counterstained with
35   4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) to identify all nuclei. The
     stained sections were examined and photographed using the Zeiss Axiovision 3.1 program
     in conjunction with Zeiss Axioskop™ 2 microscope, and analyzed using Image J (NIH).

5   Tumors from the neurosphere cell population were large and well-circumferential,
and showed cells positive for the NSC marker nestin, as well as cells positive for the
lineage markers GFAP, β-tubulin III, and myelin/oligodendrocytes. Most of the labeling
within the tumor volume was directed against GFAP, and a lesser degree of labeling was
observed for β-tubulin III, myelin/oligodendrocyte, and nestin. A significant portion (>

10   75%) of the tumor volume stained positive for reticulin, consistent with a sarcomatous
component. Additionally, H&E staining revealed a high grade glioma with necrosis
consistent with a glioblastoma, displaying the dual nature of the gliosarcoma.
Neurospheres formed high grade gliomas with necrosis as seen on H&E (FIG. 5A). The
tumors were large and well circumferential as evidenced in non-stained sections (FIG. 5B)

15   and stained for the nuclear marker DAPI (FIG. 5D). A comparison of non-tumor area
(FIG. 5D) with tumor area (FIG. 5E) stained for reticulin revealed high levels of reticulin
in tumor engulfed regions, showing the histological sarcomatous component of the
gliosarcoma. The staining patterns suggest that 9L neurospheres recapitulated the original
tumor by differentiating into both neural and glial lineages *in vivo*.

20

Example 4. Proliferation Rate and Drug Sensitivity of 9L Neurospheres

       To compare the differential proliferation rates and resistance to chemotherapeutic
agents, 2,000 healthy 9L sphere and monolayer cells were exposed to either Dulbecco's
modified Eagle's medium/F-12 medium containing 10% fetal bovine serum (FBS), or 100

25   μM stock solution of Temozolamide or Carboplatin dissolved in PBS at concentrations of
1,000 μM, 500 μM, 250 μM, and 125 μM for 2 days. The viability of the cells was scored
by measurement of the absorption of formazan dye (the amount of formazan dye formed
directly correlates to the number of metabolically active cells) using the cellular
proliferation assay WST-1 (Roche Molecular Biochemicals, Mannheim, Germany).

30   Formazan was measured with the use of a microplate reader (Tecan) and
spectrophotometer set at a wavelength of 440 nm and a reference wavelength of 890 nm.
Cellular viability was determined by exposing cells to WST-1 for 4 hours, and calculating
the percentage of viable cells. Proliferation was also assessed by using manual cell
counting after 7 days in culture, with an initial cellular concentration of 100,000 cells/mL

35   in a 25 mm^2 flask.

       The neurospheres demonstrated significantly ($P < 0.05$) greater resistance to
temozolamide (FIG. 6B) and carboplatin (FIG. 6A) when compared to the 9L cells grown
as a monolayer under the same conditions. When the cells were not treated with the

33

5   chemotherapeutic agents and simply grown in medium, there was a significantly ($P < 0.05$)

greater increase in cell number in the monolayer group by a factor of 1.48 when using the

WST-1 proliferation assay when the cells were not treated with the chemotherapeutic

agents. The results of the proliferation assays are depicted in FIG. 7, which is a bar graph

that shows a greater increase in cells in the monolayer group. A similar trend in the

10 untreated cells was also observed using the manual cell count method, which showed a

greater increase in monolayers by a factor of 1.35. This example demonstrates that

neurosphere cells have a greater resistance to chemotherapeutic agents.

Example 5. Isolation of Human CD133-Positive Cancer Stem Cells

15   Glioblastoma specimens were obtained from patients (with informed consent) via

the Brain Tumor Registry and were reviewed and released by a pathologist in the operating

room. Independent pathologists classified the tumors by type and grade in accordance

with the WHO histological grading of central nervous system tumors. IRB certified

technicians processed the glioma tissues under sterile conditions in a laminar flow hood.

20 Tumor cells were cultured in the following complete medium: Ham's F-12/DMEM with

high glucose (Irvine scientific, Santa Ana, CA), 10 mM HEPES (Invitrogen, Carlsbad,

CA), 0.1 mg/ml Gentamicin (Invitrogen) and 10% heat-inactivated FBS (Irvine Scientific,

Santa Ana, CA). The cultured cells were maintained for 3-4 passages. Floating

neurosphere-like cells were obtained that were capable of forming new spheres in medium

25 containing FBS for 3-4 passages.

            Three adult glioblastoma primary tumor cell lines (Nos. 1049, 377, and 66) were

derived by the above method and analyzed by FACS for CD133 expression. Tumor cells

were collected and stained with anti-CD133 antibody (mouse monoclonal IgG1; 1: 10;

Milteny Biotec) or IgG1 isotype control antibody (BD Pharmingen, San Diego, CA).

30 After PE-anti-mouse IgG1 (BD Pharmingen) staining for 30 minutes, CD133 staining was

analyzed by flow cytometry using a FACSCalibur™ fluorescence activated cell sorter

(Becton Dickinson, San Jose, CA). CD133 expression was observed in 10.2% (No. 66;

FIG. 9C), 27% (No. 1049, FIG. 9A) and 69.7% (No. 377; FIG. 9B) of the total population

examined.

35   To investigate the capacity of self-renewal and clonogenic potential of CD133$^+$

cell, a single isolated CD133 positive cancer stem cell was isolated by

DAKOcytomation™ (DAKO, Carpinteria, CA) sorting and cultured in a defined serum-

free NSC medium (Kabos et al., Exp. Neurol., 178:288-293, 2002) containing 20 ng/ml of

34

5   basic fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ), 20 ng/ml of epidermal

growth factor (EGF, Peprotech) and 20 ng/ml leukemia inhibitory factor (LIF, Chemicon,

Temecula, CA). Single isolated CD133 positive cancer stem cells were able to form

neurospheres (FIG. 8A), and were demonstrated to have the capacity for self-renewal and

clonogenic potential (FIG. 8B), and sustained expression of CD133 in serum-free medium

10   containing EGF/FGF (FIG. 8C).

Example 6. Human CD133 Positive Tumor Cells Express Markers Associated With
Neural Precursors

To determine the expression of other genes in CD133 positive cells, CD133

15   positive cells and CD133 negative cells were obtained by FACS sorting as described in

Example 5 and real-time PCR was used to analyze some markers associated with neural

precursors in these two populations. Total RNA was extracted from the isolated CD133

positive and CD133 negative cells using an RNA4PCR™ kit (Ambion, Austin, TX)

according to the manufacturer's protocol. For cDNA synthesis, ~1 μg total RNA was

20   reverse-transcribed into cDNA using Oligo dT primer and iScript™ cDNA synthesis kit

reverse transcriptase. cDNA was stored at -20 °C. Gene expression was quantified by

real-time quantitative RT-PCR using QuantiTect™ SYBR Green dye (Qiagen, Valencia,

CA). DNA amplification was carried out using Icycler™ (BIO-RAD, Hercules, CA), and

the detection was performed by measuring the binding of the fluorescence dye SYBR

25   Green I to double-stranded DNA. All the primer sets were obtained from Qiagen (see

Table 1).

Table 2. Oligonucleotide primers sequences used for SYBR Green real-time PCR

Gene	Forward	Reverse
Beta-actin	5'-TTCTACAATGAGCTGCGTGTG-3' (SEQ ID NO:1)	5'-GGGGTGTTGAAGGTCTCAAA-3' (SEQ ID NO:2)
CD133	5'-GCATTGGCATCTTCTATGGTT-3' (SEQ ID NO:3)	5'-CGCCTTGTCCTTGGTAGTGT-3' (SEQ ID NO:4)
MSI1	5'-GAGACTGACGCGCCCCAGCC-3' (SEQ ID NO:5)	5'-CGCCTGGTCCATGAAAGTGACG-3' (SEQ ID NO:6)
MELK	5'-CTTGGATCAGAGGCAGATGTTTGGAG-3' (SEQ ID NO:7)	5'-GTTGTAATCTTGCATGATCCAGG-3' (SEQ ID NO:8)

PSP	5'-GGCGGGGCAGTGCCTTTCAAA-3' (SEQ ID NO:9)	5'-TGTTGGCTGCGTCTCATCAAAACC-3' (SEQ ID NO:10)
CD90	5'-CGCTCTCCTGCTAACAGTCTT-3' (SEQ ID NO:11)	5'-CAGGCTGAACTCGTACTGGA-3' (SEQ ID NO:12)
NESTIN	5-ATCGCTCAGGTCCTGGAA-3' (SEQ ID NO:13)	5'-AAGCTGAGGGAAGTCTTGGA-3' (SEQ ID NO:14)
CD44	5'-AGAAGGTGTGGGCAGAAGAA-3' (SEQ ID NO:15)	5'-AAATGCACCATTTCCTGAGA-3' (SEQ ID NO:16)
GLI1	5'-AGGGAGGAAAGCAGACTGAC-3' (SEQ ID NO:17)	5'-CCAGTCATTTCCACACCACT-3' (SEQ ID NO:18)
CXCR4	5'-GATCAGCATCGACTCCTTCA-3' (SEQ ID NO:19)	5'-GGCTCCAAGGAAAGCATAGA-3' (SEQ ID NO:20)
Bmi-1	5'-GGAGACCAGCAAGTATTGTCCTTTTG-3' (SEQ ID NO:21)	5'-CATTGCTGCTGGGCATCGTAAG-3' (SEQ ID NO:22)
PTCH1	5'-CGCCTATGCCTGTCTAACCATGC-3' (SEQ ID NO:23)	5'-AAATGGCAAAACCTGAGTTG-3' (SEQ ID NO:24)
Snail	5'-ACCACTATGCCGCGCTCTT-3' (SEQ ID NO:25)	5'-GGTCGTAGGGCTGCTGGAA-3' (SEQ ID NO:26)
SIRT1	5'-ACTTGTACGACGAAGACGAC-3' (SEQ ID NO:27)	5'-CAGAAGGTTATCTCGGTACC-3' (SEQ ID NO:28)
Survivin	5'-TGCCTGGCAGCCCTTTC-3' (SEQ ID NO:29)	5'-CCTCCAAGAAGGGCCAGTTC-3' (SEQ ID NO:30)
CIAP1	5'-CAGCCTGAGCAGCTTGCAA-3' (SEQ ID NO:31)	5'-CAAGCCACCATCACAACAAAA-3' (SEQ ID NO:32)
CIAP2	5'-TCCGTCAAGTTCAAGCCAGTT-3' (SEQ ID NO:33)	5'-TCTCCTGGGCTGTCTGATGTG-3' (SEQ ID NO:34)
NAIP	5'-GCTTCACAGCGCATCGAA-3' (SEQ ID NO:35)	5'-GCTGGGCGGATGCTTTC-3' (SEQ ID NO:36)
XIAP	5'-AGTGGTAGTCCTGTTTCAGCATCA-3' (SEQ ID NO:37)	5'-CCGCACGGTATCTCCTTCA-3' (SEQ ID NO:38)
BCL-2	5'-CATGCTGGGGCCGTACAG-3' (SEQ ID NO:39)	5'-GAACCGGCACCTGCACAC-3' (SEQ ID NO:40)
BCL-X_L	5'-TGCATTGTTCCCATAGAGTTCCA-3' (SEQ ID NO:41)	5'-CCTGAATGACCACCTAGAGCCTT-3' (SEQ ID NO:42)
FLIP	5'-CATCCACAGAATAGACCTGAAGACAA-3' (SEQ ID NO:43)	5'- GCTTGGAGAACATTCCTGTAACTTG-3' (SEQ ID NO:44)

BAX	5'-TGG AGCTGCAGAGGATGATTG-3' (SEQ ID NO:45)	5'-GAAGTTGCCGTCAGAAAACATG-3' (SEQ ID NO:46)
BCRP-1	5'-TGGCTGTCATGGCTTCAGTA-3' (SEQ ID NO:47)	5'-GCCACGTGATTCTTCCACAA-3' (SEQ ID NO:48)
MGMT	5'-CTGGCTGAATGCCTACTTCC-3' (SEQ ID NO:49)	5'- CAACCTTCAGCAGCTTCCAT-3' (SEQ ID NO:50)
OCT4	5'-CCTGAAGCAGAAGAGGATCA-3' (SEQ ID NO:51)	5'-CCGCAGCTTACACATGTTCT-3' (SEQ ID NO:52)

5

Quantification of target gene mRNA as compared to an internal control (beta-actin) was performed by following a ΔC_T method. An amplification plot that had been the plot of fluorescence signal vs. cycle number was drawn. The difference (ΔC_T) between the mean values in the duplicated samples of target gene and those of beta-actin were

10 calculated by Microsoft Excel and the relative quantified value (RQV) was expressed as $2^{-\Delta CT}$. The relative expression of each gene was compared to autologous CD133 negative cells. The results of the QT-PCT analysis are presented in Table 3.

Table 3. Relative Expression of Genes in CD133+ Cancer Stem Cells

	No. 66		No. 377		No. 1049	
Gene name	**CD133–**	**CD133+**	**CD133–**	**CD133+**	**CD133–**	**CD133+**
CD90	1	15.6 ± 0.66	1	12.8 ± 0.94	1	13.5 ± 0.75
CD44	1	5.7 ± 0.48	1	2.5 ± 0.22	1	2.8 ± 0.19
CXCR4	1	337.8 ± 29.2	1	251.5 ± 22.1	1	264.9 ± 22.9
Nestin	1	21.4 ± 1.25	1	23.2 ± 1.65	1	22.1 ± 1.54
MSI	1	84 ± 7.6	1	75.4 ± 7.03	1	53.5 ± 6.2
MELK	1	1351 ± 95.8	1	467.7 ± 40.5	1	514.6 ± 45.6
GLI-1	1	46 ± 3.8	1	43 ± 4.5	1	49 ± 5.9
PTCH	1	16 ± 1.48	1	13.5 ± 0.85	1	14.3 ± 1.24
MGMT	1	32.4 ± 2.5	1	34.7 ± 2.9	1	56.3 ± 4.2
BCRP1	1	6.5 ± 0.43	1	4.3 ± 0.25	1	4.8 ± 0.24
SIRT1	1	4.9 ± 0.34	1	4.2 ± 0.26	1	5.4 ± 0.29
FLIP	1	294 ± 25.5	1	157.6 ±	1	145.6 ±

				14.2		13.7
BCL-2	1	13.9 ± 0.95	1	4.9 ± 0.54	1	3.8 ± 0.54
BCL-XL	1	5.6 ± 0.39	1	3.2 ± 0.16	1	2.5 ± 0.14
cIAP1	1	39.0 ± 3.5	1	4.3 ± 0.53	1	5.6 ± 0.65
cIAP2	1	3 ± 0.25	1	1.9 ± 0.12	1	1.7 ± 0.14
XIAP	1	21.9 ± 2.2	1	9.7 ± 0.68	1	10.3 ± 0.91
NAIP	1	12.1 ± 0.75	1	6.4 ± 0.43	1	4.5 ± 0.62
Survivin	1	1.6 ± 0.08	1	2.3 ± 0.18	1	2.4 ± 0.18
BAX	1	0.33 ± 0.03	1	0.49 ± 0.06	1	0.21 ± 0.05

5

CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1), and maternal embryonic leucine
zipper kinase (MELK) mRNA expression on CD133 positive cancer stem cells was
upregulated by an average of 15.6, 5.7, 337.8, 2.14, 84, and 1351 fold, respectively,
compared to the levels found on autologous CD133 negative tumor cells. mRNA levels

10   for GLI1 and PTCH1 were upregulated an average of 46 and 16 times, respectively, in
CD133 positive cells, as compared to CD133 negative cells. Furthermore, Bmi-1,
phosphoserine phosphatase (PSP), SHH, OCT4 and Snail mRNA were expressed in
CD133 positive cells derived from the three cell lines; none of the five genes were
detectable on CD133 negative cells.   Additionally, anti-apoptotic genes were also

15   upregulated (see Example 8).

Example 7. CD133 Positive Cancer Stem Cells Are Resistant to Chemotherapeutic Agents

To determine whether CD133 positive cancer stem cells were resistant to
chemotherapeutic agents, the WST-1 Cell Proliferation Assay was used to examine the

20   drug sensitivity of CD133 positive cells and CD133 negative cells (both collected by
FACS sorting from the three glioblastoma patients' primary cultured tumor cells as
described above). CD133 positive and negative cells were exposed to conventional
chemotherapeutic agents, temozolomide, carboplatin, VP-16 or taxol at various
concentrations, for up to 48 hours in 10% FBS/F-12/DMEM culture medium.

25   Temozolomide was supplied by the Schering-Plough Research Institute (Kenilworth, NJ)
and was dissolved in DMSO (Sigma Chemical Co., St Louis, MO) at 100 mM stock
solution. Carboplatin, etoposide (VP-16) and paclitaxel (Taxol) were obtained from
Sigma-Aldrich (St. Louis, MO). CD133 positive cells isolated from No. 66 showed

5   dramatic drug resistance to the above four agents including temozolomide (FIG. 10C),
       carboplatin (FIG. 10D), VP-16 (FIG. 10A), and Taxol (FIG. 10B) as compared to
       autologous CD133 negative cells. CD133 positive cells isolated from No. 377 showed
       significant resistance to carboplatin at all concentrations tested (FIG. 11A) and to VP-16 at
       200 μM (FIG. 11B). CD133 positive cells isolated from No. 1049 showed significant
10   resistance to carboplatin at 200 μM compared to autologous CD133 negative cells (FIG.
       11C). This example demonstrates increased drug resistance of CD133 positive cancer
       stem cells as compared to autologous CD133 negative cells.

       Example 8. Anti-Apoptotic Genes Are Upregulated in CD133 Positive Cancer Stem Cells
15   Real-time PCR of FACs-sorted CD133 positive and CD133 negative cells was used
       to investigate the relative expression of multi-drug resistance genes and genes related to
       inhibiting cell apoptosis between these two populations. BCRP1 has been demonstrated to
       play an important role in the drug resistance of normal stem cells and tumor stem cells
       (Zhou et al., Nat. Med., 7:1028-34, 2001; Hirschmann-Jax et al., Proc. Natl. Acad. Sci.
20   USA, 101:14228-33, 2004). Higher expression of BCRP1 (6.5 fold) was found in CD133
       positive cells as compared to that of autologous CD133 negative cells. Furthermore, anti-
       apoptotic genes, such as FLIP, BCL-2, and BCL-XL, were found at significantly higher
       levels (294, 13.9 and 5.6 times higher) in CD133 positive cells than in CD133 negative
       cells. Also, inhibitor of apoptosis protein (IAPs) family members, XIAP, cIAP1, cIAP2,
25   NAIP, and survivin were found at higher expression levels on CD133 positive cells 21.9,
       39.04, 3.03, 12.1, 6.73, and 1.6 times higher, respectively, than in CD133 negative cells. It
       has been demonstrated that SIRT1 deacetylates the DNA repair factor Ku70, causing it to
       sequester the pro-apoptotic factor, Bax, away from mitochondria, thereby inhibiting stress-
       induced apoptotic cell death (Cohen et al., Science, 305:390-392, 2004). SIRT1
30   deacetylase mRNA expression was increased 4.92 times in CD133 positive cells. The pro-
       apoptotic gene BAX was decreased 3 times in CD133 positive cells as compared to
       autologous CD133 negative cells. Thus, gene expression differences were observed in
       CD133 positive cells as compared to CD133 negative cells.

35   Example 9. Recurrent Glioblastomas Express Higher Levels of CD133
               Malignant glioma is a highly recurrent tumor even after surgery, chemotherapy,
       radiation and immunotherapy. To address the potential role of CD133 positive tumor cells
       in glioblastoma recurrence, the CD133 expression upon first and second resection of tumor

5 tissue from the same patient were compared. We obtained primary tumor tissue from five

pathological confirmed grade IV astrocytoma (GBM) patients, and then re-operated

following radiation, chemotherapy, and/or immunotherapy to obtain recurrent tumor

tissue. RNA extraction and RT-PCR was performed for CD133 as described above. For

all tested patients, CD133 expression was significantly higher in recurrent tumor tissue

10 (relative expression ~2.5-19.1) than that in autologous primary tumor tissue (relative

expression ~1)(FIG. 12). This example implicates a resistant CD133 positive tumor

population in tumor recurrence.

Example 10. Immunization With Cancer Stem Cell Antigens Increased Survival

15   A dendritic cell vaccine was generated using antigens from neurospheres, daughter

cells, and monolayer cells. Immature dendritic cells were generated from the bone marrow

of 6-12-week-old Fisher F344 rats as previously described (Talmor et al., Eur. J.

Immunol., 28:811-817, 1998). Briefly, bone marrow was harvested from the femoral and

tibial marrow cavities and cultured in RPMI 1640 media supplemented with 10% fetal

20 bovine serum (Gemini Biotechnologies, Calabasas, CA), 1% Penicillin/Streptomycin

(Invitrogen, Carlsbad, CA), 50 ng/ml recombinant rat GM-CSF and 100 ng/ml

recombinant rat IL-4 (R & D Systems, Minneapolis, MN). Cultures were fed every 2 days

by removing 75% of the media and replacing it with fresh media containing cytokines (this

washed away most of the lymphocytes and granulocytes). To determine the percentage of

25 immature dendritic cells generated and cultured from the bone marrow of Fisher rats after

1 week of exposure to GM-CSF and IL-4, FACS analysis was run on cells immunostained

with antibodies for CD86 (costimulatory marker B7-1; DC marker), CD80 (costimulatory

marker B7-2; DC marker), CD3 (T cell marker), or MHC II (DC marker). The dendritic

cell populations obtained were positive for CD86, CD80, and MHC II, while negative for

30 CD3.

Soluble peptides were generated for dendritic cell pulsing by cell lysis. 9L

neurospheres, daughter cells, and monolayer (adherent) cells were processed in the

laboratory to produce a single cell suspension. The cells were then lysed by 4 to 5 freeze

cycles (on liquid nitrogen) and thaw cycles (room temperature). Lysis was monitored by

35 light microscopy, and larger particles were removed by centrifugation (10 minutes at 600×

g). The supernatants were passed through a 0.2 µm filter, and protein concentration was

determined by BioRad protein assay and aliquots frozen at −80 °C until use.

5   To establish intracranial tumors, adult Fisher F344 rats were stereotactically

inoculated in the right corpus striatum (from bregma in mm: anterior-posterior +1 mm;

medial-lateral –3 mm; dorsal-ventral –5 mm) with 25,000 9L-luciferase tumor cells as

described above. For vaccination of the rats, freshly cultured immature dendritic cells

were cocultured overnight for 24 hours with 80-100 μg of cell lysate one day prior to the

10   vaccination at days 7, 14, and 21 post-operatively. Vaccinations were given

subcutaneously in the flanks on days 7, 14, and 21 with 50,000 DCs pulsed with antigens

from either 9L neurospheres (NS), daughter cells (DtC), monolayer (adherent) cells (AC),

or saline control. The animals were followed for survival and euthanized when terminal

neurological signs developed, for example, inability to access food, water, seizure activity,

15   weakness, and paralysis.

Tumor bearing rats injected with three successive vaccines (once per week) of

dendritic cells pulsed with control, AC , DtC, or NS antigens, had median survival dates of

26.5, 32, 29 and 50 days, respectively (FIG. 13). Kaplan-Meier analysis showed that rats

treated with 9L neurosphere lysate-pulsed DC had significantly longer survival time than

20   each of the other groups (p = 0.0015).

Example 11. DCs Pulsed With Cancer Stem Cell Antigens Induced a Strong Cytotoxic T

Cell Response Against 9L Tumor Cells

To determine whether the relative protective effect of NS-DC vaccination on

25   survival is due to tumor-specific immunity, a cytotoxic T lymphocyte (CTL) assay was

performed. Spleens were removed on day 28 post-intracranial 9L-luciferase tumor cells

implantation from groups of rats treated with either control or 9L peptide–pulsed dendritic

cells. Splenocytes were isolated and re-stimulated in vitro, as described (Wunderlich et al.,

"Assays for T-cell function," In: Coligan et al., eds., Current Protocols in Immunology,

30   New York, NY, John Wiley & Sons, Inc; 1997:3.11.1–3.11.20; Ehtesham et al., J.

Immunother., 26:107–116, 2003) with either irradiated (10,000 rads) 9L adherent cells

(AC), daughter cells (DtC) or neurospheres (NS). The effector: target (E:T) ratio was 6:1.

Re-stimulated cells were cultured in RPMI-1640 with 10% fetal bovine serum, 1%

penicillin-streptomycin, and 1% HEPES in 6-well flat-bottom plates in a humidified 37 °C,

35   5% CO_2 incubator. Cells were incubated for 11 days with addition of IL-2 (300 units/mL)

every 3 days. After 11 days of re-stimulation, the cells were re-exposed to their initial

target (i.e., irradiated 9L AC, DtC, or NS). 24 hours later, culture media was harvested

from the remaining cells and used for IFNγ protein quantification by ELISA (Ehtesham et

5   al., J. Immunother., 26:107–116, 2003). Re-stimulated splenocytes from rats treated with
       NS pulsed DCs showed significantly higher IFNγ release in response to exposure to tumor
       cell targets than re-stimulated splenocytes from rats treated with AC or DtC (FIG. 14),
       indicating that a higher cytotoxic T cell response was obtained in the NS-DC vaccinated
       rats. These data were also confirmed by PCR for IFNγ levels (data not shown). The
10   higher IFNγ response in the NS-DC vaccinated group corresponds to the higher survival
       rate observed in the same group. These data show that a cancer stem cell antigen vaccine
       targets tumor cells more potently than vaccines based on tumor antigens from daughter
       cells, or cell populations that are not enriched for cancer stem cells.

15   Example 12. Intracranial T-Cell Infiltration Is Associated With Prolonged Survival
              A representative number of brains from DC-DtC and DC-NS vaccinated rats were
       carefully removed and post-fixed in 4% paraformaldehyde. Coronal sections of 20 µm
       were cut on a cryostat and blocked with normal horse serum for 1 hour. Slides were then
       incubated with anti-CD4 (clone OX-38 monoclonal antibody diluted in 1:200 in PBS) for 2
20   hours at room temperature, followed by a 20 minute incubation at room temperature with
       the linking antibody (BioGenex biotinylated anti-mouse immunoglobulin). After washing
       in PBS, the labeled moiety (BioGenex Horse Radish Peroxidase-conjugated streptavidin)
       was added for 20 minutes at room temperature. DAB (3,3'-diaminobenzidine) was used as
       the chromogen. For analysis of CD8 expression, slides were incubated overnight with
25   Anti-CD8 alpha Chain, clone OX-8 antibody (Chemicon).
              Immunohistochemical assessment of brain sections from rats vaccinated with
       dendritic cells pulsed with NS vaccinated showed that there was a robust infiltration of
       CD4^{+} (FIG. 15B) and CD8^{+} lymphocytes that was not observed in the brain sections
       obtained from rats vaccinated with dendritic cells pulsed with daughter cell antigens (FIG.
30   15A). This infiltration of T cells correlates with increased survival of rats vaccinated with
       NS pulsed DCs.
       Example 13. Cancer Stem Cells Express MHC, Unlike Normal Stem Cells
              Five glioblastoma multiforme (GBM) cancer stem cells (CSC) were generated by
       the methods described in Yuan et al. (Oncogene, 23:9392-9400, 2004). Briefly, tumor
35   specimens were obtained within half an hour of surgical resection from five adult GBM
       patients, as approved by the Institutional Review Boards at the Cedars-Sinai Medical
       Center. Tumor tissue was washed, minced, and enzymatically dissociated (Reynolds et al.,

5   Science, 255:1707-10, 1992). Tumor cells were resuspended in DMEM/F12 medium

containing 10% fetal bovine serum (FBS) as growth medium and plated at a density of

2×10^6 live cells per 75 cm² flask. The cells attached and grew as a monolayer in flasks.

All the five monolayer growing adult GBM cells were switched into a defined serum-free

NSC medium (Reynolds et al., Science, 255:1707-10, 1992) containing 20 ng/ml of basic

10   fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ) and 20 ng/ml of epidermal

growth factor (EGF, Peprotech). Normal human fetal neural stem cells (NSC) were

cultured in the same defined serum-free medium as for cancer stem cells. CSC and NSC

cells were stained by FITC-HLA-A,B,C antibody and isotype control antibody (BD

Bioscience, San Diego, CA) and analyzed by flow cytometry. Representative CSC and

15   NSC results were shown in the FIGs. 17A-17B and 18A-18B, respectively.

HLA expression was seen in 5 of 5 cancer stem cells from different patients. CSCs

expressed high levels of HLA-A,B,C, however, NSC did not expression MHC class I

(HLA-A,B,C) antigens on the surface. This unexpected result indicates that specific

cancer stem cell antigens on cancer stem cells can be targeted using vaccines that generate

20   T cells that recognize and kill cancer stem cell antigens in the context of MHC. Therefore

cancer stem cells will be targeted, whereas normal stem cells will not.

Example 14. Isolation of CD133 T Cell Epitopes

A nucleic acid encoding a portion of CD133 extracellular domain 1 (amino acid

25   residues 116-270 of SEQ ID NO:53) is used in cloning and expression on the surface of

DC. DC are transfected with CD133-1 cDNA construct or empty vector (mock) for 48

hours. The successful transfection of DC-CD133 cells is identified either by anti-CD133

monoclonal antibody using flow cytometry or by EGFP cloned vector under fluorescent

microscopy.

30   Antigen presenting cells with CD133 receptor are stimulated by CD133 T cell

epitopes. Overlapping peptides of 8-10 amino acids of residues 325-350 of SEQ ID

NO:53 are produced as MHC class I epitopes. Similarly, overlapping peptides of 13-20

amino acid of residues 325-350 are produced as MHC class II epitopes. Stimulated APC

using the peptide epitopes lead to enhanced production of CD8 T cells targeted to CD133

35   molecular on stem/progenitor cells in brain tumors.

5

OTHER EMBODIMENTS

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

10

5   **WHAT IS CLAIMED IS:**

1. A method of treating neural cancer in a patient, the method comprising:
    obtaining a population of dendritic cells;
    contacting the dendritic cells with a neural cancer stem cell antigen composition
under conditions such that the dendritic cells present neural cancer stem cell antigens; and
10   administering to a patient a composition comprising the dendritic cells.

2. The method of claim 1, wherein the dendritic cells are autologous.

3. The method of claim 1, wherein the dendritic cells are allogeneic.

15

4. The method of claim 1, wherein the neural cancer stem cell antigen composition is
obtained from a brain tumor.

5. The method of claim 4, wherein the brain tumor is a glioma.

20

6. The method of claim 1, wherein the neural cancer stem cell antigen composition
comprises a lysate of neural cancer stem cells.

7. The method of claim 1, wherein the neural cancer stem cell antigen composition
25   comprises an acid eluate of neural cancer stem cells.

8. The method of claim 6, wherein the neural cancer stem cells express CD133.

9. The method of claim 1, wherein the neural cancer stem cell antigen composition
30   comprises one or more isolated peptides of CD133, CD90, CD44, CXCR4, Nestin,
Musashi-1 (Msi1), maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1,
Bmi-1, phosphoserine phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP,
BCL-XL, XIAP, cIAP1, cIAP2, NAIP, or survivin.

35   10. The method of claim 9, wherein the one or more isolated peptides are synthetic.

5   11. A method of preparing a neural cancer vaccine, the method comprising:

obtaining a population of dendritic cells; and

contacting the dendritic cells with a neural cancer stem cell antigen composition

under conditions such that the dendritic cells present neural cancer stem cell antigens, thus

preparing a neural cancer vaccine.

10

12. A method of preparing a cell vaccine for treating a neural cancer, the method

comprising:

obtaining mononuclear cells from a subject;

culturing the mononuclear cells in vitro under conditions in which mononuclear

15   cells differentiate into antigen presenting cells;

isolating neural cancer stem cells from the subject;

obtaining a neural cancer stem cell antigen composition from the neural cancer

stem cells; and

culturing the antigen presenting cells in the presence of the neural cancer stem cell

20   antigen composition, thus preparing a cell vaccine.

13. The method of claim 12, further comprising administering the cell vaccine to the

subject.

25   14. A kit for preparing a cell vaccine for treating a brain cancer, the kit comprising:

one or more isolated peptides of CD133, CD90, CD44, CXCR4, Nestin, Musashi-1

(Msi1), maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1,

phosphoserine phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL,

XIAP, cIAP1, cIAP2, NAIP, or survivin.

30

15. A composition consisting essentially of dendritic cells comprising one type or one or

more different types of neural cancer stem cell antigens.

16. The composition of claim 15, wherein the neural cancer stem cell antigens include

35   peptides of one or more of CD133, CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1),

maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine

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5   phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1,
cIAP2, NAIP, or survivin.

17. A method of treating cancer in a patient, the method comprising:
        obtaining a population of dendritic cells;
10   contacting the dendritic cells with a cancer stem cell antigen composition under
conditions such that the dendritic cells present cancer stem cell antigens; and
        administering to a patient a composition comprising the dendritic cells.

18. A method of preparing a cancer vaccine, the method comprising:
15   obtaining a population of dendritic cells; and
        contacting the dendritic cells with a cancer stem cell antigen composition under
conditions such that the dendritic cells present cancer stem cell antigens, thus preparing a
cancer vaccine.

20   19. A method of preparing a cell vaccine for treating a cancer, the method comprising:
        obtaining mononuclear cells from a subject;
        culturing the mononuclear cells in vitro under conditions in which mononuclear
cells differentiate into antigen presenting cells;
        isolating cancer stem cells from the subject;
25   obtaining a cancer stem cell antigen composition from the cancer stem cells; and
        culturing the antigen presenting cells in the presence of the cancer stem cell antigen
composition, thus preparing a cell vaccine.

20. A method of treating cancer in a patient, the method comprising:
30   administering to a patient a composition consisting essentially of dendritic cells
that present cancer stem cell antigens.

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FIG. 1

Generation of immature
dendritic cells

Pulsing of dendritic cells
with BTSC antigens

Mature antigen-presenting
dendritic cells

Antigen
preparation

+ IL-4
+ GM-CSF

Tumor excision
and isolation of
brain tumor
stem-like cells
(BTSC)

Vaccination of the patient

Isolation of monocytes from
peripheral blood

FIG. 2

Tumor excision

Enzymatic dissociation

Plating monocellular suspension in serum containing medium

Establishment of adherent cells

Switch with a medium containing EGF and bFGF

Tumor Neurospheres formation

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E
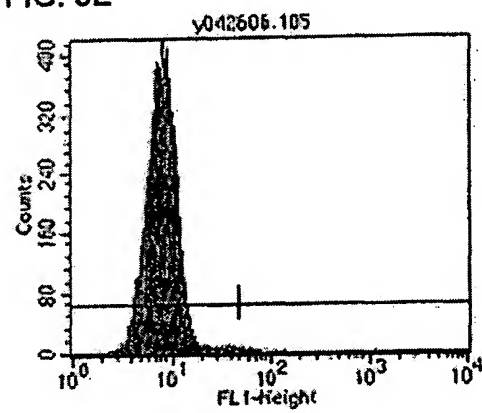
FIG. 3F
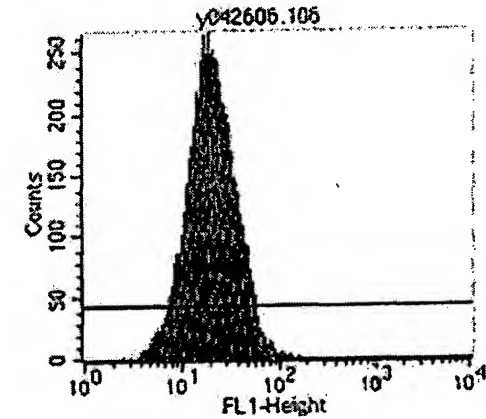
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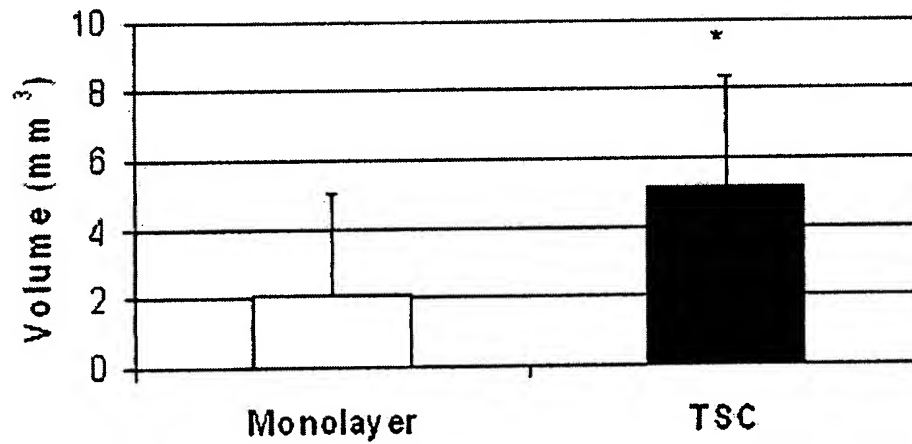
FIG. 4B
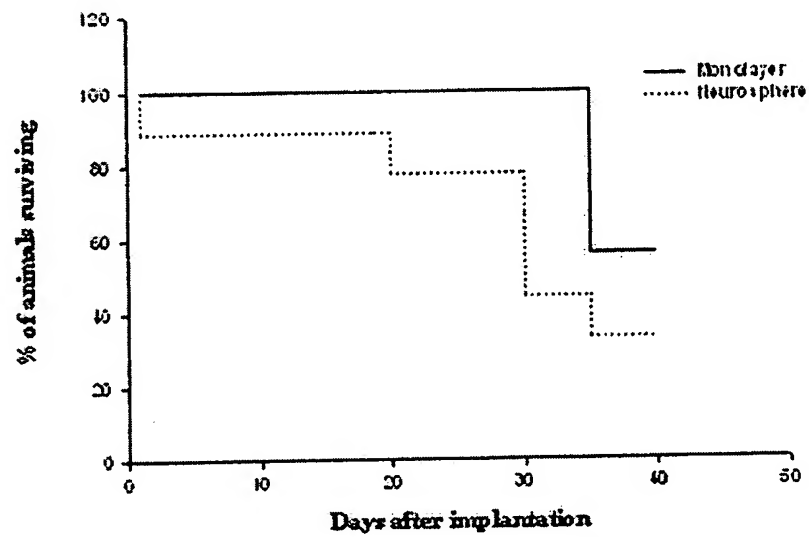
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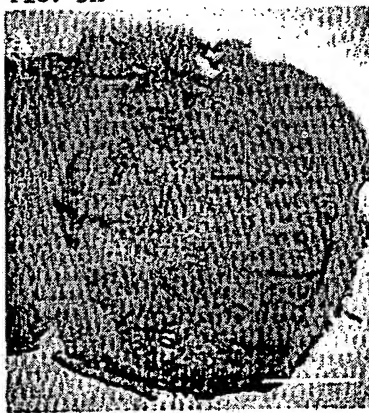
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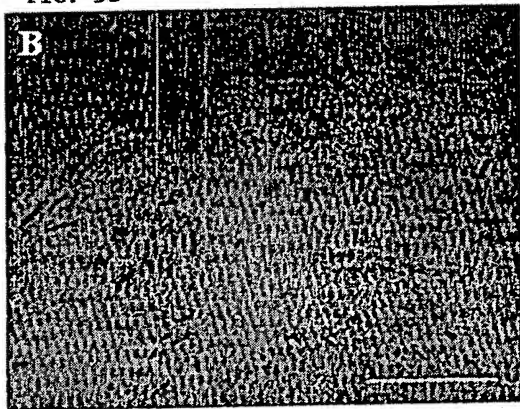
FIG. 5C
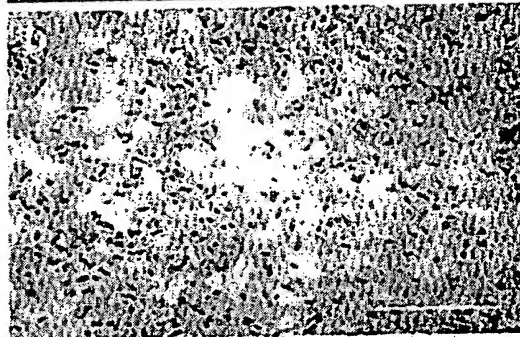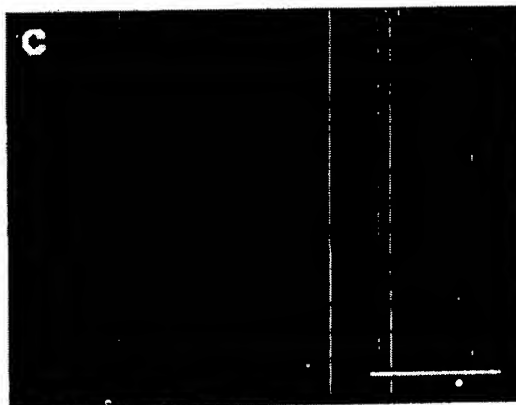
FIG. 5D
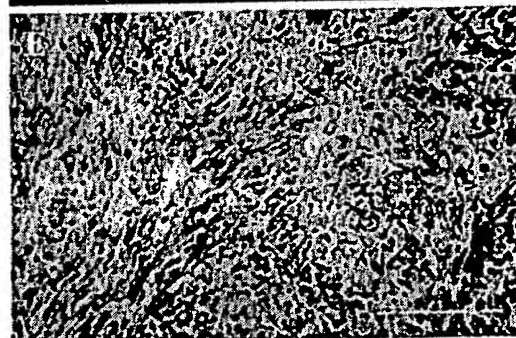
FIG. 5E

FIG. 6A
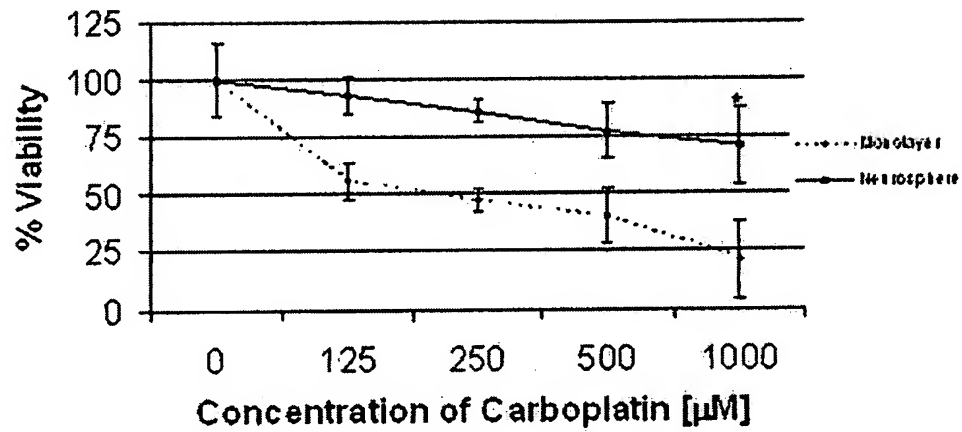
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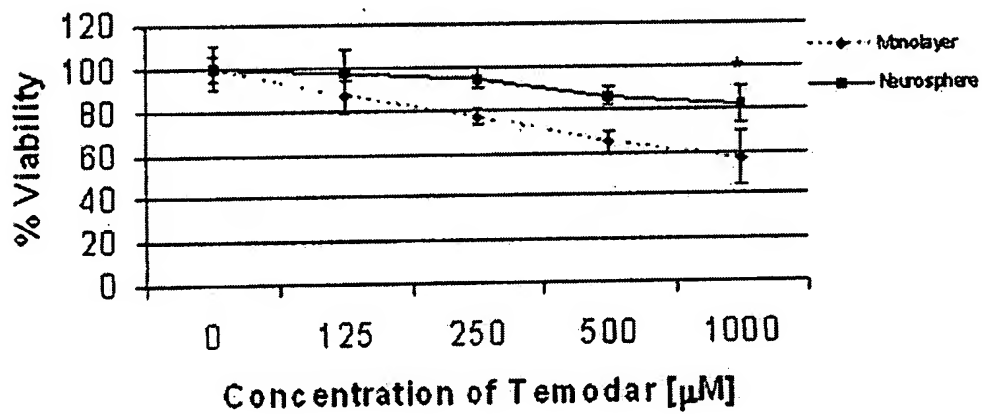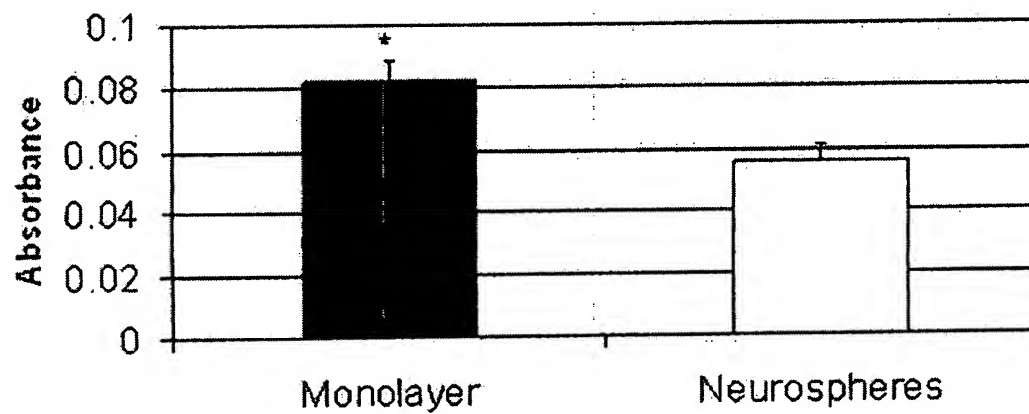
FIG. 7

FIG. 8A
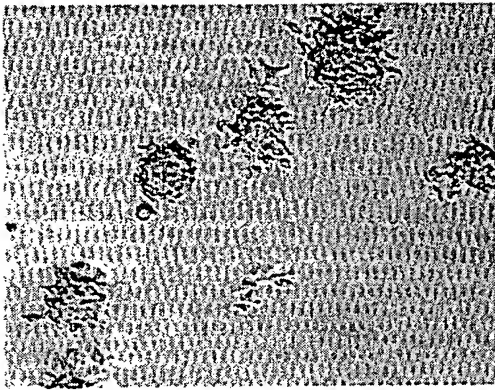
FIG. 8B
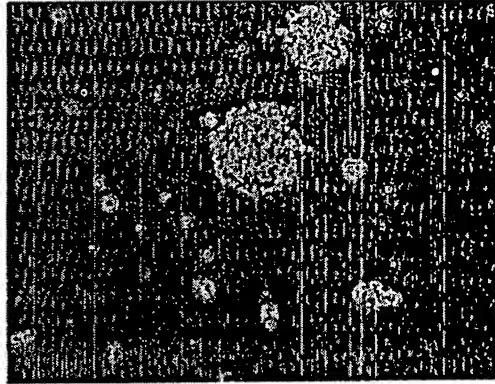
FIG. 8C
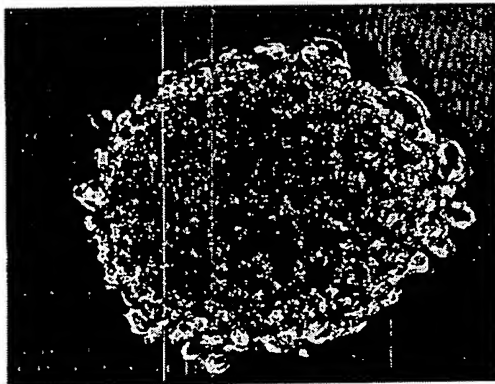
FIG. 9A
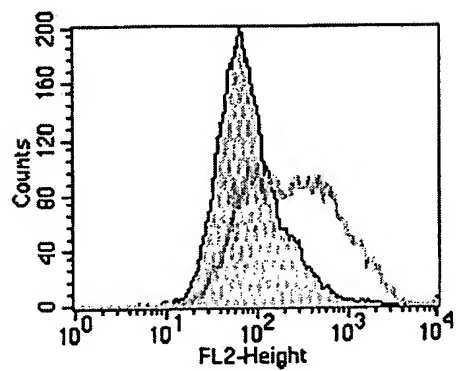
FIG. 9B
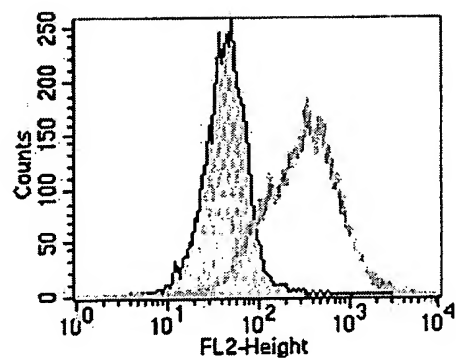
FIG. 9C
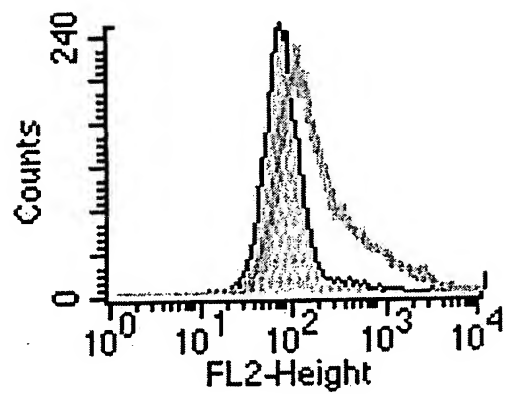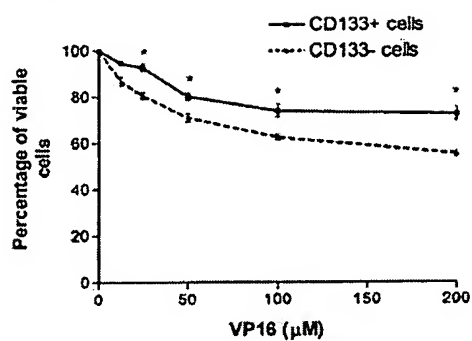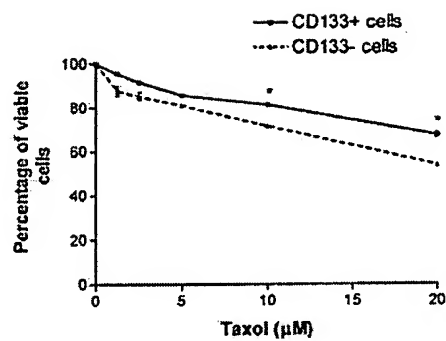
FIG. 10A

FIG. 10B

FIG. 10C
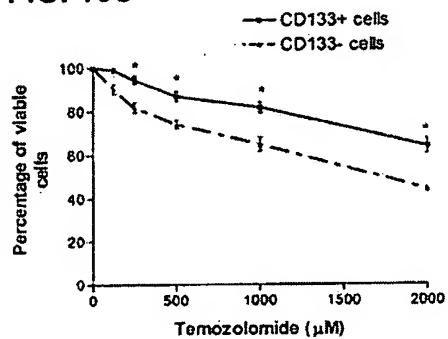
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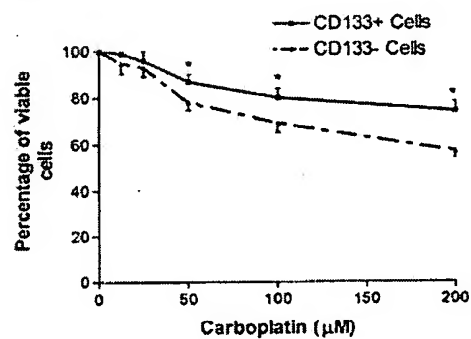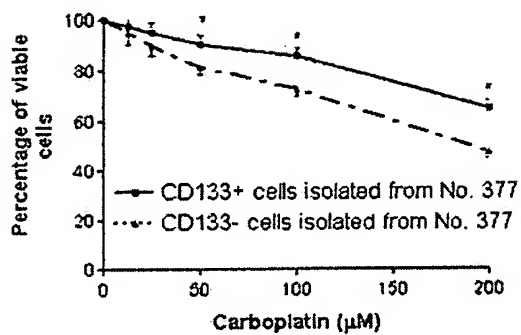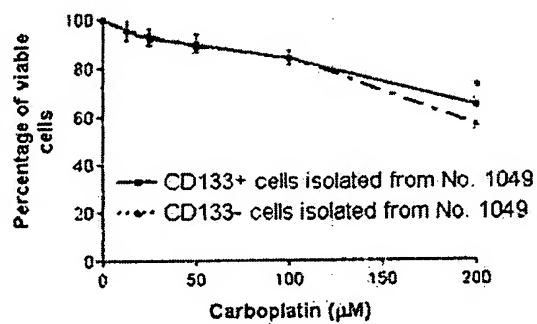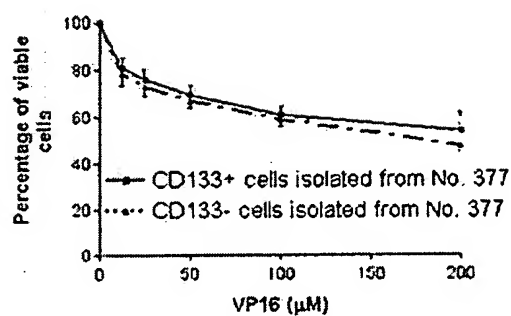
FIG. 11A

FIG. 11B

FIG. 11C

FIG. 12
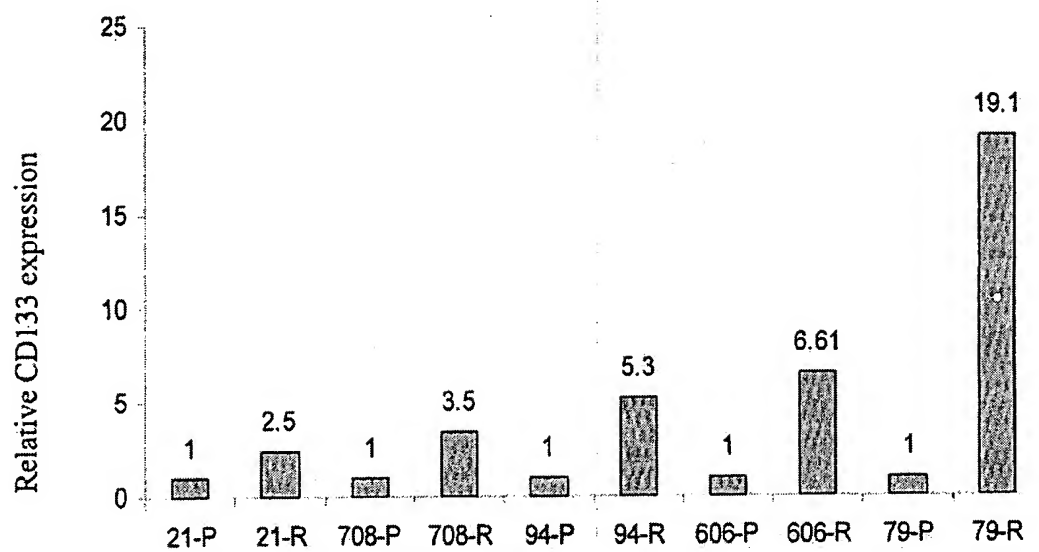
FIG. 13
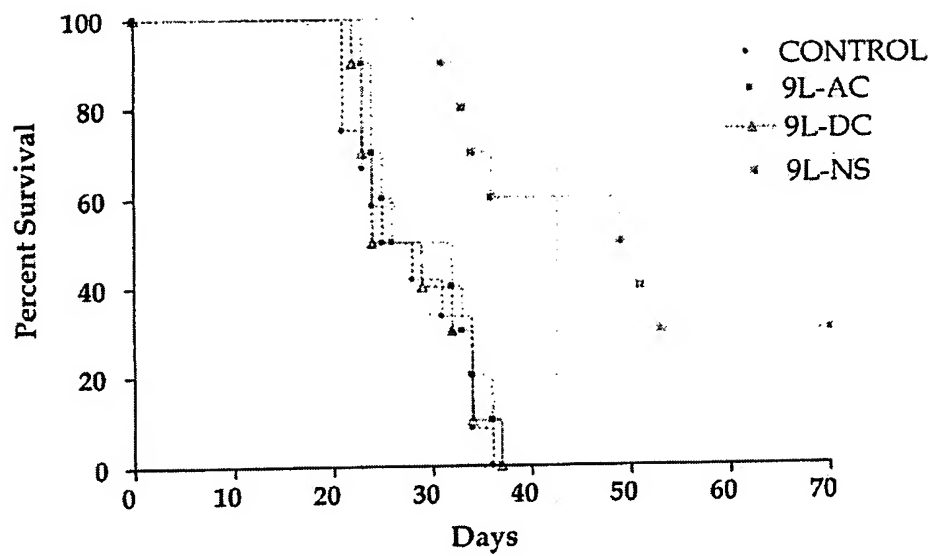
FIG. 14
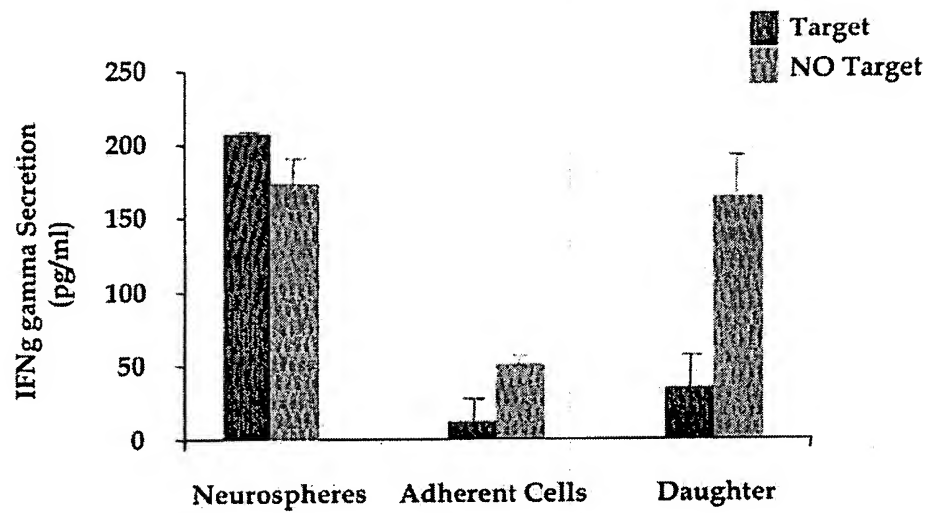
FIG. 15A
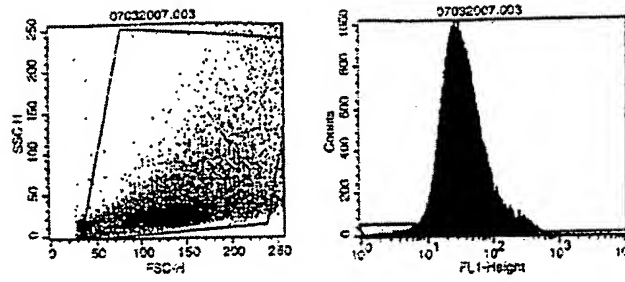

FIG. 15B
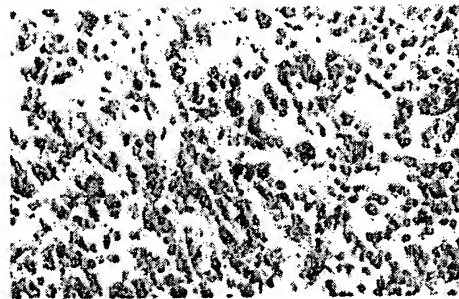
FIG. 16
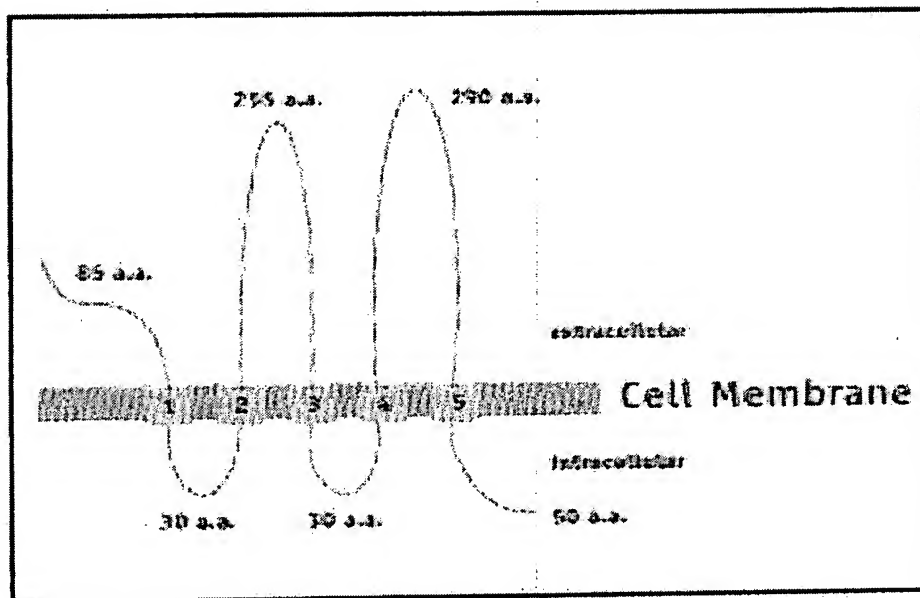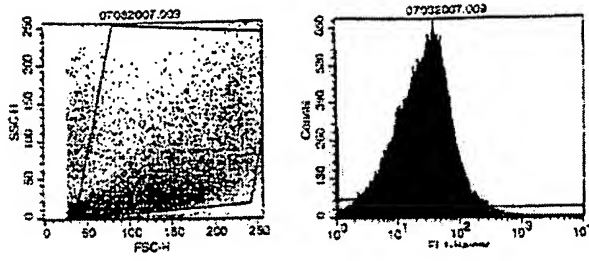
FIG. 17A

FIG. 17B

17/17

FIG. 18A

FIG. 18B
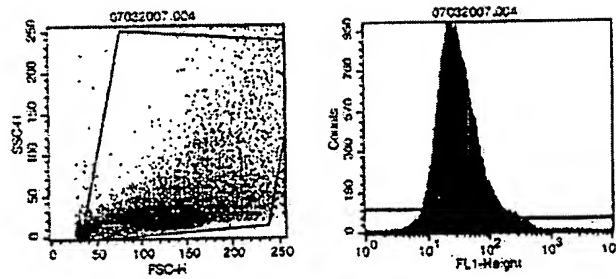
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26 September 2007 (26.09.2007)

(25) Filing Language: English

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60/826,955   26 September 2006 (26.09.2006)   US

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(75) Inventor/Applicant *(for US only)*: YU, John, S. [US/US]; 86 West Third Street, #800E, Los Angeles, CA 90048 (US).

(74) Agents: FASSE, Peter, J. et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(88) Date of publication of the international search report:
10 July 2008

(54) Title: CANCER STEM CELL ANTIGEN VACCINES AND METHODS
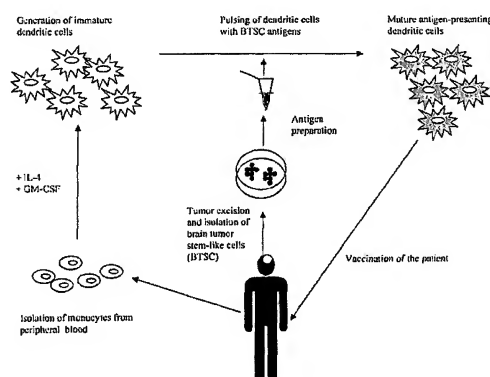
(57) Abstract: Method of stimulating an immune response (*e.g.*, to treat cancer) include administering to a patient a composition including dendritic cells that present cancer stem cell antigens. Compositions including cancer stem cell antigens are also provided herein.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/06 (2008.01)
USPC - 435/325, 435/335

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 435/325, 435/335

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
C12N 5/06 (2008.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB) and Google Scholar
Search Terms Used:
dendritic ADJ cell ADJ vaccination, DC ADJ vaccination, brain ADJ cancer, cancer ADJ vaccine, neural ADJ cancer ADJ antigen, neural ADJ cancer ADJ stem ADJ cell ADJ antigen, antigen-presenting ADJ cell, mononuclear ADJ cell, dendritic ADJ cell, CD13

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GILBAO et al. Immunotherapy of cancer with dendritic-cell-based vaccines. Cancer Immunology and Immunotherapy. 1998 Vol 46, pages 82-87 especially Abstract (pg 82), page 83 col 2 para 1, page 83 col 2 para 1 and 2, page 84 col 1 para 1 and 2.	17-20
Y		1-16
Y	US 20060204509 A1 (HARTY et al.) 14 September 2006 (14.09.2006) para 0044, 0061, 0069, 0053, 0070 and 0121.	1-16
Y	SINGH et al. Identification of a Cancer Stem Cell in Human Brain Tumors. Cancer Research, September 15, 2003 Vol 63, pages 5821?5828 especially Abstract (pg 5821) and page 5822 col 2 para 1.	8-10, 14 and 16

☐ Further documents are listed in the continuation of Box C.　☐

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2008 (21.012008)	2 7 MAR 2008

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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